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THE UNIVERSITY OF ALBERTA

INHIBITION OF UREASE BY HETEROCYCLIC MERCAPTANS

by

Ⓒ WILLIAM DOUGLAS GOULD

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Inhibition of Urease by Heterocyclic Mercaptans" submitted by William Douglas Gould in partial fulfilment of the requirements for the degree of Doctor of Philosphy in Soil Microbiology.

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ABSTRACT

The inhibition of jack bean urease by a number of heterocyclic mercaptans was investigated. The following compounds not previously tested as urease inhibitors were found to be effective inhibitors: 1,3,4-thiadiazole-2,5-dithiol; 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione; 5-amino-1,3,4-thiadiazole-2-thiol; and rhodanine. The latter two compounds required preoxidation with hydrogen peroxide before they were effective. Inhibition of urease by these heterocyclic mercaptans was reversed by reducing agents. The inhibitory properties of the heterocyclic mercaptans were found to be due to varying amounts of the corresponding disulfides or polysulfides in the mercaptan preparations. The results are consistent with the hypothesis of a thiol-disulfide exchange reaction between one or more molecules of either a disulfide or polysulfide and one or more of the sulfhydryl groups of urease.

The disulfides of 5-amino-1,3,4-thiadiazole-2-thiol and 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione were prepared and their properties investigated. They were found to be very potent inhibitors of jack bean urease. Spectroscopic studies of the mercaptans and the corresponding disulfides provided information regarding the tautomeric conformations of the mercaptans under various conditions.

The inhibition of soil urease activity by heterocyclic mercaptans and a number of other compounds was also studied. Hydroquinone and 1,4-benzoquinone were the most effective soil urease inhibitors but would be unsuitable for practical use because they are strong irritants. The disulfides of the heterocyclic

mercaptans, which were potent inhibitors of jack bean urease *in vitro*, were not very effective as soil urease inhibitors.

1,3,4-Thiadiazole-2,5-dithiol is not as toxic as the benzoquinones and is also an effective soil urease inhibitor. In laboratory experiments with soil, 1,3,4-thiadiazole-2,5-dithiol when applied at 100 ppm of soil caused a 46% reduction in soil urease activity. This compound is the most satisfactory one for field tests with fertilizer urea.

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LIST OF ABBREVIATIONS AND DEFINITIONS

K_M	Michaelis constant
K_{sp}	solubility-product constant
E^o	standard electrode potential (unit activities of oxidant and reductant)
$E^{o\prime}$	formal electrode potential (unit concentration ratio of oxidant and reductant)
E_h	redox potential (experimentally measured)
nm	nanometers
cm^{-1}	wave number (^{1}/wavelength, used in infrared spectroscopy)
MH_z	megahertz (10^6 cycles per second)
m/e	mass/charge ratio (each peak in a mass spectrum is characterized by its m/e ratio)
λmax	wavelength at which maximum absorption occurs
ϵ	molar extinction coefficient (Beer's law: optical density = ϵlc)
n.m.r.	nuclear magnetic resonance spectroscopy
TMS	tetramethylsilane (an internal standard for n.m.r. spectroscopy)
DMSO	dimethyl sulfoxide
$DMSO_{d6}$	deuterated dimethyl sulfoxide

δ or ppm	the chemical shift of a proton in the n.m.r. spectrum $= \dfrac{\Delta\mu(\text{cps})\ \text{X}\ 10^{6}}{\text{oscillator frequency (cps)}}$
cps	cycles per second
$\Delta\mu$	the difference between the absorption frequency of a proton in the n.m.r. spectrum and the absorption frequency of the internal standard (usually TMS)
Parent Peak	in mass spectrometry the parent peak is the one which corresponds to the molecular ion. The m/e value of the molecular ion is equal to the molecular weight
Base Peak	the most intense peak in a mass spectrum
M.P.	melting point
CEC	cation exchange capacity
meq	milliequivalents
TRIS	tris (hydroxymethyl) aminomethane
EDTA	(ethylenedinitrilo) tetraacetic acid
BSA	bovine serum albumen
NADH	reduced nicotinamide adenine dinucleotide
ATP	adenosine triphosphate
ϕ	phenyl group
KBr	potassium bromide

WSCD	water-soluble carbodiimide. In this study the following one was used: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
SU	Sumner unit. One Sumner unit is the amount of urease that liberates one milligram of ammonia N from a 3% urea solution at pH 7.0, and 20°C in 5 minutes.
IU	International unit. One International unit is the amount of urease that catalyzes the decomposition of one μequivalent of the bond involved in one minute (usually defined at 30°C, although 20°C can be used). 1 SU (20°C) = 14.28 IU (20°C)
I_{50}	the molar concentration of inhibitor at which a urease preparation has 50% of its activity remaining
A.O.A.C.	Association of Official Agricultural Chemists

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INTRODUCTION

The ability to control the rate of hydrolysis of urea by the enzyme urease has practical implications in both medicine and agriculture. It is well known that urea itself is relatively non-toxic but the free ammonia that results as one of the products of urea hydrolysis certainly is toxic.

Urease inhibitors could be of value in treating hyper-ammonaemic states by reducing the contribution of gastric urease to blood ammonia levels resulting from infections of ureolytic bacteria (Fishbein, 1967). Chemical urease inhibitors administered to patients in hepatic coma have been shown to lower blood ammonia levels but evaluation of the clinical benefit of this treatment is still under investigation (Summerskill _et al_, 1967).

In agriculture, non-protein nitrogen sources have become popular as feed supplements for ruminants because they are cheaper than conventional protein sources. Urea is used as a non-protein nitrogen source, but it can only be used in limited quantities because of problems arising from the rapid hydrolysis of urea in the rumen. Urea is hydrolyzed to ammonium bicarbonate more rapidly than the ammonium ions can be used by the rumen microorganisms. A large portion of the excess ammonium is absorbed across the rumen wall into the blood stream of the animal, resulting in both toxicity and the inefficient use of nitrogen. Larger quantities of urea could be incorporated into urea feeds if rumen urease activity could be reduced (Jones, 1968).

Urea is now one of the important nitrogenous fertilizers. At high rates of urea application, particularly on calcareous soils of low buffering capacity, there can be poor crop responses. Several factors are responsible for some of the problems encountered when urea is used and the primary ones are the loss of nitrogen by volatilization of ammonia gas, and toxicity to plants caused by the ammonia produced by the hydrolysis of urea. These problems could be eliminated if the hydrolysis rate of urea was controlled. The rate at which ammonium bicarbonate is produced from urea in the soil could be retarded by either decreasing the rate at which urea dissolves in the soil solution or by reducing soil urease activity. A number of slow release urea fertilizers are now on the market such as: urea-formaldehyde polymers, sulfur-coated urea, and various other types of coatings and mixtures (Beaton _et al_, 1967, Lunt, 1971, Mays and Terman, 1969). A large number of compounds have also been evaluated as soil urease inhibitors with a view to decreasing the rate at which urea fertilizer hydrolyzes in the soil.

The initial objective of this research was to assess the effectiveness of a number of chemicals as soil urease inhibitors. A new class of urease inhibitors, the heterocyclic mercaptans, were discovered during the initial experiments. Subsequently, additional work was carried out to delineate the mechanism by which these compounds inhibit urease. The heterocyclic mercaptans that were studied in detail were: 1,3,4-thiadiazole-2,5-dithiol, 5-amino-1,3,4-thiadiazole-2-thiol, 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione, and 2-thioxo-4-thiadiazolidinone (rhodanine).

LITERATURE REVIEW

I. Distribution of Urease

The enzyme urease (urea amidohydrolase, EC No. 3.5.1.5) which catalyzes the hydrolysis of urea was the first enzyme to be purified, and was first crystallized from the jack bean (Canavalia ensiformis) by Sumner (1926). Urease is found in some higher plants and in many species of bacteria, yeasts, and fungi (Sumner, 1953). Although urease was the first enzyme to be purified, its properties and mechanism are still not fully understood.

In biological systems urea is formed by the enzymatic degradation of purines, and by the urea-ornithine cycle (Kamin and Handler, 1957, Reinbothe and Mothes, 1962). Urease serves differing functions in various organisms. Citrullus seeds contain high concentrations of urease which functions as a storage protein to be consumed during germination (Williams, 1950). In some microorganisms urease is involved in nitrogen metabolism, particularly when nitrogen is limiting (Kaltwasser et al, 1972). It was originally thought that urea was a required nitrogen source for T-strain mycoplasmas and urease was necessary to break down urea so it could be used in nitrogen metabolism (Ford and MacDonald, 1967). However, more recent work indicates that these mycoplasmas can use several other nitrogen sources (Ford et al, 1970, Masover and Hayflick, 1973).

In both plants and microorganisms the synthesis of urease is frequently induced by its substrate urea and repressed by the product ammonia (Durzan, 1973, Magana-Plaza and Ruiz-Herrera, 1967, Matsumoto et al, 1968, Shim et al, 1973, Stewart, 1965). Several species of Pseudomonas, Hydrogenomonas, and Micrococcus synthesize large quantities of urease under conditions of nitrogen starvation (Kaltwasser et al, 1972). It is not clear whether urea is an inducer or a "non-repressor nitrogen source" and thus causes "derepression" due to lack of nitrogen precursors of the repressor protein. Kaltwasser suggested that ammonia or amino acids can function as precursors of repressor protein. It is also possible that urea could be present as an inducer during nitrogen starvation, since the endogenous metabolism of purines will produce urea (De Turk, 1955). Sporosarcina ureae and Proteus vulgaris have urease as a constitutive enzyme since both produce large amounts of it under all culture conditions (Kaltwasser et al, 1972).

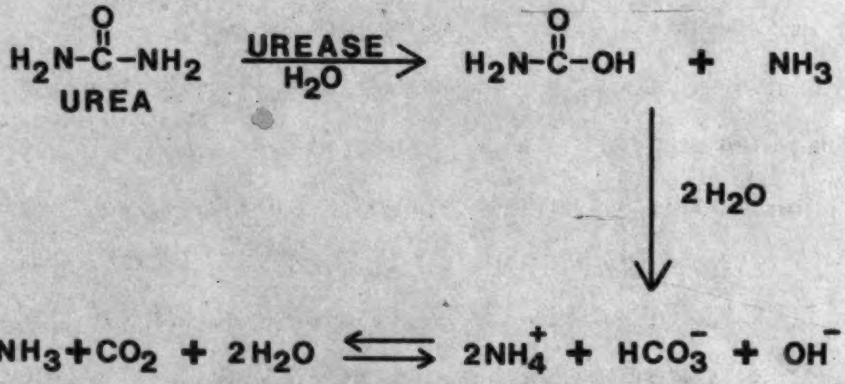
Some yeasts and unicellular algae can utilize urea as a nitrogen source but do not possess urease (Cook and Boulter, 1964, Leftley and Syrett, 1973, Roon and Levenberg, 1968). These organisms have been found to possess a new enzyme capable of hyrolyzing urea called ATP: urea amidolyase, which is an ATP dependent biotin requiring enzyme (Roon and Levenberg, 1968, 1970). ATP: urea amidolyase is either two separate enzymes (Thompson and Muenster, 1971) or an enzyme complex (Whitney and Cooper, 1972). The hydrolysis is in two steps; the binding of bicarbonate and urea followed by the release of allophanate; and then the hydrolysis of allophanate to ammonium bicarbonate (Roon and Levenberg, 1970, Whitney and Cooper, 1970). Urea amidolyase has a lower K_M for urea than urease and may function in environments where urea concentrations are very low (Roon and Levenberg, 1972).

II. Properties of Urease

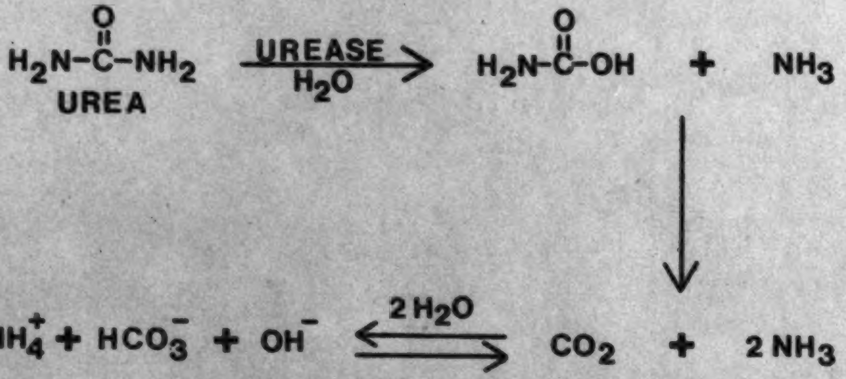
Most of the studies of the kinetics and properties of urease have been with jack bean urease. Although urease was once considered specific for urea it has been shown more recently to be capable of hydrolyzing hydroxyurea (Fishbein et al, 1965), dihydroxyurea (Fishbein, 1969b) and semicarbazide (Gazzola et al, 1973).

The final end product of urea hydrolysis is ammonium bicarbonate. The sequence of products is most likely one of the three following possibilities (Varner, 1960).

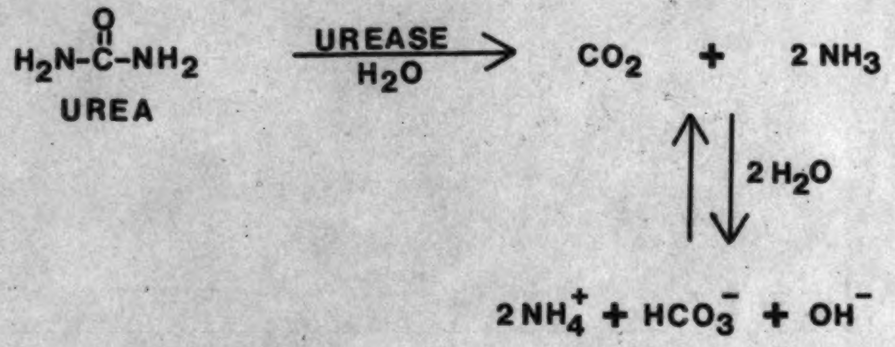
1.


H2N-C(=O)-NH2
UREA
UREASE
H2O
H2N-C(=O)-OH
+
NH3
2 H2O
2 NH3 + CO2 + 2 H2O
2NH4+ + HCO3- + OH-

2.


H2N-C(=O)-NH2
UREA
UREASE
H2O
H2N-C(=O)-OH
+
NH3
2NH4+ + HCO3- + OH-
2 H2O
CO2
+
2 NH3

3.



$$\underset{\text{UREA}}{H_2N\text{-}\overset{O}{\overset{\|}{C}}\text{-}NH_2} \xrightarrow[H_2O]{\text{UREASE}} CO_2 + 2\,NH_3 \overset{2\,H_2O}{\rightleftharpoons} 2\,NH_4^+ + HCO_3^- + OH^-$$

Isotope studies of urea hydrolysis in H_2O^{18} enriched water eliminate reaction sequence 1 as a possibility but do not indicate which of sequences 2 or 3 are correct (Wang and Tarr, 1955). Although carbamic acid has been shown to be present in the reaction mixture during the hydrolysis of urea by urease (Sumner et al, 1931), it is possible that carbamic acid can be formed from ammonia and carbon dioxide. Gorin (1959) used carbonic anhydrase to prevent the formation of carbamate from ammonia and carbon dioxide, and was able to show that carbamate is present during the hydrolysis of urea. The initial kinetic parameters for the hydrolysis of urea at limiting substrate concentrations are consistent with carbamic acid and ammonia as first reaction products (Blakeley et al, 1969). Thus, reaction sequence 2, which involves the enzymatic cleavage of urea to ammonia and carbamic acid, followed by the chemical hydrolysis of carbamic acid to ammonia and carbon dioxide is the currently accepted mechanism for the complete reaction in the urease-catalyzed hydrolysis of urea.

Urease activity is affected by pH, buffer, salt, and urea concentrations (Howell and Sumner, 1934). Fasman and Niemann (1951) investigated the effects of phosphate buffers on urease activity, and found sodium and potassium ions inhibited urease, and phosphate behaved as an activator. Kistiakowsky et al (1952) suggested that the inhibitory species are complexes of either sodium or potassium with the various phosphate anions in solution.

The optimum pH for urease activity is pH 7.0 in maleate buffer (Kistiakowsky and Rosenberg, 1952) and pH 8.0 in tris (hydroxymethyl) aminomethane (TRIS)-sulfate buffer (Wall and Laidler, 1953a). The difference in the pH optima between various buffer systems and other anomalous results have been clarified somewhat by more recent work. In citrate and TRIS buffers the end product is ammonium carbamate whereas phosphate and maleate buffers catalyze the chemical hydrolysis of carbamate to ammonia and carbon dioxide (Jespersen, 1975). The ammonium ion concentration resulting from the hydrolysis of urea would be higher in the phosphate and maleate buffers than in the citrate and TRIS buffers. The ammonium ion has been shown to inhibit urease (Hoare and Laidler, 1950). Thus, most of the differences between various buffer systems can be attributed to the differences in the end products of urea hydrolysis.

Fischgold (1934) found urease activity to be independent of oxidation-reduction potential (E_h) over a fairly wide range, but Sizer and Tytell (1941) found the activity of jack bean urease to vary with E_h. Some of the compounds Sizer and Tytell used to poise the E_h can inhibit urease, so their results are in question.

The variation of enzyme activity with enzyme concentration should be linear when the substrate is in excess. Urease activity has been shown by some workers to vary linearly with enzyme concentration (Kistiakowsky et al, 1952) and others have found a nonlinear relationship (Wall and Laidler, 1953b, Peterson et al, 1948). Wall and Laidler (1953a) found the kinetics of urea hydrolysis by urease to follow the Michaelis-Menten scheme with the rate falling off somewhat at high substrate concentrations. Conflicting results were reported by Kistiakowsky and Rosenberg (1952) which indicated that urease deviates from Michaelis-Menten kinetics at both high and low substrate concentrations. In order to account for these deviations they proposed the existence of either two types of active sites with differing Michaelis constants or identical sites which interact with each other. The Michaelis constant of urease is independent of ionic strength (Kistiakowsky and Thompson, 1956). The first step of urea hydrolysis involves the reaction of an uncharged urea molecule with the enzyme, so the magnitude of the dissociation constant for the enzyme-substrate complex should not vary with ionic strength. Thus, the observed Michaelis constant is a true one. Carbon and nitrogen kinetic isotope effects have been demonstration for urease (Rabinowitz et al, 1956, Singleton et al, 1951). Lynn and Yankwich (1962) studied C^{13} isotope effects in detail and concluded that the mechanism is very complex. Lynn (1967) found the temperature dependence of urease to vary with the enzyme preparation used, and although Michaelis-Menten kinetics were followed, each preparation had a unique pair of kinetic parameters. Lynn and Yankwich (1964) suggested that urease preparations contain a mixture of isozymes and interconversions between the isozymes are responsible for the kinetic complexity. Subsequent work by other authors, which will be discussed later, has confirmed this hypothesis.

The rates of enzyme-catalyzed reactions tend to increase with temperature until at higher temperatures the rate decreases rapidly due to denaturation of the enzyme (Ashmore, 1963). Over intermediate temperature ranges the temperature dependence of most enzymes can be described by the Arrhenius equation:

$$K = A\ e^{-Ea/RT}$$

K = rate constant

A = pre-exponential factor

Ea = activation energy

R = gas constant

T = temperature in °K

The activation energy of an enzyme is dependent on the experimental conditions. Activation energies varying from 4.4 kcal/mole to 11.7 kcal/mole have been reported for urease (Larson and Kallio, 1954, Miller et al, 1968, Sizer, 1943). Sizer (1943) reported the existence of a temperature discontinuity in the Arrhenius plot of urease, but this observation has been disputed by others (Kistiakowsky and Lumry, 1949, Miller et al, 1968). The anomalous temperature effects are probably caused by changes in the distribution of the various isomeric forms of urease under different experimental conditions, thus resulting in corresponding changes in the temperature dependence of urease activity.

The molecular weight of urease has been determined many times and typical values are 483,000 (Sumner et al, 1938) and 487,000 (Siegel and Monty, 1965). The isoelectric point of urease has been shown to be between pH 4.9 and pH 5.1 (Contaxis and Reithel, 1971a, Creeth and Nichol, 1960, Sumner and Hand, 1929). Two isoelectric points of pH 4.75 and 5.15 have been reported for different components of a mixture of urease isozymes (Fishbein and Nagarajan, 1972a). Recent work indicates that urease is a nickel metalloenzyme and contains nine atoms of nickel per urease molecule (Dixon et al, 1975). The exact function of the nickel atoms in urease has not yet been clarified.

The presence of sulfhydryl groups in urease was first demonstrated by Sumner and Poland (1933), and sulfhydryl groups have since been shown to be essential for the catalytic activity of urease. Hellerman et al (1943) found three types of sulfhydryl groups; 20 - 22 groups per urease molecule that are highly reactive but not necessary for enzymic activity, another 20 - 22 that are somewhat less reactive but essential for enzymic activity, and 60 relatively unreactive groups that are not involved in enzyme activity. However, the reagents they used to titrate sulfhydryl groups have been criticized for either not reacting completely or being nonspecific (Cecil and McPhee, 1959, Gorin et al, 1962). More recent work indicates that there are 26 - 28 reactive sulfhydryl groups per urease molecule, another 7 - 9 that are necessary for enzymic activity and 50 that can only be titrated after urease has been exposed to 6.0 M guanidine hydrochloride (Andrews and Reithel, 1970). Guanidine hydrochloride causes urease to unfold so the sulfhydryl groups in the interior of the enzyme are accessible to the titration reagent.

Titration of urease activity with specific urease inhibitors and reagents specific for sulfhydryl groups have been used to determine the number of active sites per urease molecule. The results have varied from two to eight active sites per molecule. Titration of urease with N-ethylmaleimide indicates eight active sites per molecule (Andrews and Reithel, 1970, Gorin and Chin, 1965) and with silver ions three to four (Ambrose et al, 1951). Two moles of caprylohydroxamic acid will completely inhibit one mole of urease (Kobashi et al, 1966) and inactivation of urease by deuterons indicates four to six active sites (Setlow, 1952).

Many proteins of molecular weight over 50,000 - 100,000 are stable assemblies of structural subunits. Structural subunits of molecular weight 50,000 - 60,000 (Gorin et al, 1967) and 80,000 are formed when urease is treated with sodium dodecyl sulfate. Guanidine hydrochloride will dissociate urease into subunits of molecular weight 83,300 (Reithel et al, 1964), and other dissociating agents give subunits of 60,000 (Blattler and Reithel, 1970), 30,000 and 90,000 (Contaxis and Reithel, 1972). End group analysis and peptide composition imply a structural subunit of molecular weight 75,000 (Bailey and Boulter, 1969).

A number of studies have been carried out to determine the smallest subunit that still possesses catalytic activity. Diffusion rate studies suggest that a subunit of molecular weight 17,000 may be active, although the observed activity may have resulted from an aggregation of the smaller units (Hand, 1939).

Urease can dissociate into a stable and catalytically active half-unit of molecular weight 240,000 (Sehgal and Naylor, 1966, Sehgal et al, 1965). Dissociation can be accomplished by addition of glycols (Blattler et al, 1967, Contaxis and Reithel, 1971b, Gorin et al, 1969), by high pH (Fishbein and Nagarajan, 1972b), low pH (Gorin et al, 1968) and high ionic strength (Lynn, 1970). Both hydrophobic (Contaxis and Reithel, 1971b) and electrostatic (Fishbein and Nagarajan, 1972b) forces have been postulated to explain the bonding between the two half-units. Urease extracted from Bacillus pasteurii only exists in the half-unit form (Tanis and Naylor, 1968).

The presence of polymeric forms of urease in some preparations has been observed many times (Kuff et al, 1955, McLaren et al, 1948, Reithel and Robbins, 1967, Sumner et al, 1938). The polymeric forms of urease are held together by intermolecular disulfide bonds (Fishbein and Nagarajan, 1971) and the polymeric forms can be converted to the monomer by the addition of sulfite (Creeth and Nicol, 1960, Nicol and Creeth, 1963). The specific activities of each of the polymers is approximately the same as the monomer, and each of the polymers is a linear aggregate of monomers (Fishbein et al, 1970, Fishbein et al, 1973).

Using gel electrophoresis and a stain sensitive to urease concentrations approaching those used in kinetic studies, Fishbein (1969a) was able to detect at least twelve isoenzymes of urease (Fishbein et al, 1969). These included the half-unit of urease, polymers up to the pentamer, and a number of isozymes differing only slightly from the principal forms. Fishbein (1969a) suggested that the slightly differing forms of urease may be either conformational isozymes or molecules differing by a small subunit of molecular weight 20,000 or less. Two very similar isozymes of urease can be separated on DEAE-Sephadex (Lynn, 1971a). The isozyme content of urease preparations varies with the source of the enzyme and the purification procedure (Fishbein, 1969a). The conflicting kinetic results and anomalous temperature effects reported by many previous authors are quite reasonable in the light of the more recent work concerning the structural complexity of urease. Many of the early workers used heterogeneous mixtures of urease isozymes and the kinetic parameters of their preparations depended on the proportions of each of the isozymes.

III. Soil Urease

Urea can be weakly absorbed by soil although it is also simultaneously hydrolyzed to ammonium bicarbonate by soil urease (Broadbent *et al*, 1958). The primary retention mechanism by which urea is held in soil is salt formation between urea and the carboxylic groups of soil organic matter. At acid pH values urea can be protonated and behave as a cation (Broadbent and Lewis, 1964, Chin and Kroontje, 1962). Urea can complex with clays by hydrogen bonding (Mitsui and Takatoh, 1963), and also become protonated on the carbonyl group to form hemisalts in acid montmorillonites (Mortland, 1966). As well the carbonyl of urea can coordinate with various interlayer cations in montmorillonite (Farmer and Ahlrichs, 1969). The ammonium ions from the hydrolysis of urea in soil can then be oxidized by the nitrifying bacteria to nitrite and then nitrate (Pang *et al*, 1973).

The decomposition of urea in soil has been observed during the ammonification of calcium cyanamide since urea is an intermediate product in this reaction (Cowie, 1920). Gibson (1930) measured the rate of urea hydrolysis in a large number of soils and in sterile soil extract solutions inoculated with soil. He found urease in every soil studied and the rate of urea hydrolysis to be very rapid compared to other soil processes. The distribution of urease activity in various soil types is not well correlated with soil properties (McGarity and Myers, 1967) although a relationship between urease activity and organic carbon content is often observed (Conrad, 1942a, Dalal, 1975, Myers and McGarity, 1968). Urease activity is usually highest in the surface horizons of forest soils (Gibson, 1930, Roberge and Knowles, 1966, 1968), and lowest in alkaline and saline soils (Skujins and McLaren, 1969). Weak urease activity has been observed in the lower mineral horizons of various soils (Conrad, 1940c, Myers and McGarity, 1968). The ability to hydrolyze urea was found to vary from 17-71% for soil bacteria and 78-98% for soil fungi (Lloyd and Sheaffe, 1973, Roberge and Knowles, 1967). Soils that have been treated with urea for long periods of time do not have appreciably higher urease activities or percentages of ureolytic microorganisms than similar untreated soils (Lloyd and Sheaffe, 1973). However, soil urease activity can be induced by the addition of small amounts of urea (Paulson and Kurtz, 1969). The addition of easily metabolizable carbon and nitrogen sources will stimulate soil urease activity which is due to the increase in total microbial population of which the ureolytic microflora is a portion (Paulson and Kurtz, 1969).

Urease activity has been detected in soils that have been air-dried and stored for sixty years and in 9,500 year-old buried permafrost peat samples (Skujins and McLaren, 1968). Urease activity can also be observed in lyophilized urea-urease mixtures at 60% relative humidity which implies that urea could be hydrolyzed in air-dry soil (Skujins and McLaren, 1967). Conrad (1940a, 1940b) was able to show that urea hydrolysis in soil is a biochemical process. The presence of toluene and other antiseptics has only a slight effect on soil urease activity, and soil urease is also resistant to digestion by added trypsin (Conrad, 1940b, 1942b). Conrad (1940b) suggested that most of soil urease activity is extracellular and occurs as a ligno-protein complex. Paulson and Kurtz (1969) altered soil urease activity by adding various amendments and calculated from a regression equation that 79-89% of soil urease activity was extracellular and complexed by soil colloids.

If soil urease is extracellular in nature it must be stabilized and protected from proteolysis in order to remain active. The breakdown by soil microorganisms of a number of substrates such as dextrans and gelatin is not retarded by binding these substrates with kaolinite and illite, although bentonite has a slight protecting effect (Lynch and Cotnoir, 1956, Olness and Clapp, 1972). Digestion of various proteins adsorbed on clays by proteolytic enzymes occurs almost as rapidly as the same process in free solution (Ensminger and Gieseking, 1942, McLaren, 1954). The digestion of lysozyme adsorbed on kaolinite by bacteria is more rapid than the same process in solution (Estermann and McLaren, 1959). The highest degree of protection from proteolysis occurs when a protein is complexed with lignin (Estermann _et al_, 1959). Clay surfaces may actually act as a surface to concentrate the protein substrate and the proteolytic enzyme, thus allowing more rapid hydrolysis (Estermann and McLaren, 1959, McLaren and Estermann, 1956). Although small quantities of crystalline urease can be extracted from soil (Briggs and Segal, 1963), more recent work indicates that a significant amount of soil urease can be extracted as an organo-urease complex (Burns _et al_, 1972a, 1972b, McLaren _et al_, 1975). The extracted organo-urease complex was found to be resistant to digestion by pronase (Burns _et al_, 1972a).

The Michaelis constant of soil urease was determined to be 252 mM by Paulson and Kurtz (1970) but Tabatabai (1973) using a different assay technique found a range of values between 1.1 and 3.4 mM. The optimum activity of soil urease is at pH 7.0 (Vasilenko, 1962). Since most of soil urease is complexed with soil constituents it is useful to compare the properties of soil urease with urease bound to clay minerals and to other solid supports. Urease has been bound to a large variety of solid supports, such as gelatin (Bollmeier and Middleman, 1974), glass (Weetall and Hersh, 1969), and barium stearate covered glass slides (Langmuir and Schaefer, 1938). Sundaram and Crook (1971) added urease to kaolinite and found an increase in K_M with binding from 11.7 mM to 40 mM, and the pH optimum remaining unchanged at pH 6.65. The pH optimum of an enzyme would be expected to increase after binding to a clay mineral because the pH at a clay surface can be 1-2 units lower than the bulk solution (McLaren, 1962, McLaren and Packer, 1970). Urease immobilized in hydrocarbon-based liquid-surfactant membranes has a K_M of 180 mM at pH 7.0 (May and Li,

1972) and urease microencapsulated in nylon has a K_M of 3.8 mM (Sundaram, 1973). Urease bound covalently to nylon has a K_M of 3.5 mM, a pH optimum of 7.0 and an activation energy of 9.6 kcal/mole between 25 and 40°C (Sundaram and Hornby, 1970). Insolubilized urease also exhibits a greater stability than urease in solution (Riesel and Katchalski, 1964). Model enzyme-humic acid complexes have been prepared by reacting trypsin and pronase with p-benzoquinone to produce enzymatically active polymers having many characteristics in common with soil humic acids (Rowell et al, 1973).

The available evidence implies that most of soil urease resides as part of large cross-linked organic polymers, in such a manner that the stability of urease is enhanced and the entry of proteolytic enzymes is restricted, but the substrate and products can diffuse freely through the matrix.

Under certain conditions poor crop responses to the application of urea fertilizer have been reported (Court et al, 1964a, Stephen and Waid, 1963). A number of reasons have been suggested, such as toxicity from ammonia produced by urea hydrolysis, nitrite accumulation during nitrification, the presence of biuret in urea and the loss of gaseous nitrogen by several mechanisms.

An equilibrium between ammonium ions and ammonia gas occurs in aqueous solutions of ammonium salts. At pH values below seven the ammonium ion predominates, whereas above pH 8.0 free ammonia is the predominant form. Although the ammonium ion is relatively nontoxic, ammonia gas is very toxic (Warren, 1962) and in studies of excised beet root discs and beet root mitochondria, ammonia was found to interfere with the electron transport system, specifically the NADH oxidase system (Vines and Wedding, 1960).

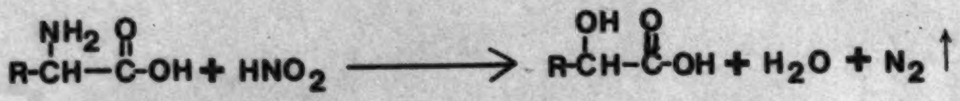
The alkaline hydrolysis products of urea can raise the pH of soil microenvironments high enough for ammonia to be volatilized and lost from the soil (Ernst and Massey, 1960). The loss of nitrogen by ammonia volatilization is favored by a high initial soil pH (Overrein and Moe, 1967) low cation exchange capacity (Martin and Chapman, 1951) and by high temperatures (Watkins et al, 1972). Ammonia losses varying from 0.4 to 80% of the applied nitrogen have been reported for agricultural soils (Gasser, 1964, Kresge and Satchell, 1960) and from 3.5 to 24.9% for forest soils (Nommik, 1973a, Overrein, 1968). Volatilization loss studies are dependent on experimental technique (Watkins et al, 1972), so the absolute magnitude of nitrogen loss may be difficult to predict but comparative studies are still valid. Some of the ammonia volatilized from urea-fertilized soil could be reabsorbed by adjacent soil (Hanawalt, 1969, Mahendrappa and Ogden, 1973). Urea has been mixed with acid materials such as phosphoric and boric acids in order to reduce volatilization losses (Bremner and Douglas, 1971b, Nommik, 1973b). Leaching losses of urea under field conditions have been found to be quite low (Overrein, 1969). The same soil conditions which enhance ammonia volatilization will also increase ammonia toxicity

Inorganic nitrogen as nitrite in the soil can be lost if the soil pH is acid via several chemical reactions as shown below:

(1) Decomposition of nitrous acid:

$$3\,HNO_2 \longrightarrow 2\,NO\uparrow + HNO_3 + H_2O$$

(2) Van-Slyke reaction involving α- amino acids:



$$R\text{-}\overset{NH_2}{\overset{|}{C}}H-\overset{O}{\overset{\|}{C}}\text{-}OH + HNO_2 \longrightarrow R\text{-}\overset{OH}{\overset{|}{C}}H\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH + H_2O + N_2\uparrow$$

(3) Decomposition of ammonium nitrite:

$$NH_4NO_2 \longrightarrow N_2\uparrow + 2\,H_2O$$

The ammonium ions produced by the hydrolysis of urea are first oxidized to nitrite by *Nitrosomonas sp.*, which are then oxidized to nitrate by *Nitrobacter spp*. For nitrite to accumulate soil conditions must be suitable for the oxidation of ammonia by *Nitrosomonas* but unsuitable for the oxidation of nitrite by *Nitrobacter* (Chapman and Liebig, 1952). Accumulation of nitrite in soil is favored by pH values of pH7 to pH8 and high ammonium ion concentrations (Hauck and Stephenson, 1965, Wetselaar *et al*, 1972). The reactions involving the volatilization of nitrogen from nitrite require low pH (Allison, 1963, Sabbe and Reed, 1964) which is not favorable for the accumulation of nitrite. Toxicity to plants from accumulated nitrite has been observed subsequent to the addition of urea fertilizer (Court et al, 1962, Court et al, 1964b).

Biuret, which interferes with protein synthesis in plants (Webster et al, 1957) can be formed by the thermal decomposition of urea during the preparation of commercial granular urea (Court et al, 1964a). The biuret content of most fertilizer urea produced currently is quite low and if the biuret content is less than 1%, little or no damage to crops results (Low and Piper, 1961, Smika and Smith, 1957, Wilkinson and Ohlrogge, 1960).

The primary problems associated with the use of urea fertilizer are nitrogen loss as ammonia and toxicity due to ammonia and nitrite.

IV. Assay of Urease Activity

The methods for determining urease activity can be grouped into two categories: (1) fixed time, and (2) continuous assays. The fixed time assays involve the reaction of urease with an excess of urea, stopping the reaction after a predetermined time period and measuring the concentration of one of the products. The concentration of base (Gorin and Chin, 1966), of ammonium (Sumner and Hand, 1928), and of $^{14}CO_2$ from ^{14}C-labelled urea (McDonald et al, 1972) have all been used as a measure of urease activity.

The continuous urease assays are: (1) potentiometric determination of the ammonium bicarbonate produced by urea hydrolysis (Katz, 1964), (2) coupling ammonium production with an ammonium requiring enzyme such as glutamic dehydrogenase (Kaltwasser and Schlegel, 1966) or horseradish peroxidase which is stimulated by ammonium (Stutts and Fridovich, 1964), and (3) using a buffer and pH indicator of similar pKa's, and spectrophotometrically following the color change of the indicator (Ruiz-Herrera and Gonzalez, 1969).

Many methods have been used to assay urease activity in soil. Soil is a heterogeneous system comprising a large number of components and a number of factors must be considered in the study of a soil enzyme. The following aspects are important in the assay of soil urease activity: (1) sampling and storage of the soil, (2) prevention of microbial growth during the assay, (3) buffer, (4) method of stopping the reaction, (5) extraction solution, (6) measurement of the amount of urea hydrolyzed.

Air-drying of soil samples and storage at either room temperature or 5°C in closed containers is the accepted pretreatment technique (Dalal, 1975, Zantua and Bremner, 1975b). A number of sterilants such as toluene (Drobnik, 1961, Roberge, 1968), a high energy electron beam (McLaren et al, 1957) and ^{60}Co radiation (Roberge and Knowles, 1968) have been used to prevent microbial activity. The most commonly used one, toluene, has some disadvantages, which can be overcome if ionizing radiation is used as a sterilant (Roberge, 1968). Soil urease assays have been performed in both the presence (Tabatabai and Bremner, 1972) and absence (Zantua and Bremner, 1975a) of buffer. Soil urease assays in the absence of buffer are more likely to resemble field conditions and also produce more consistent results (Zantua and Bremner, 1975a). Phenylmercuric acetate (Douglas and Bremner, 1970) and silver sulfate (Tabatabai and Bremner, 1972) have been used to stop the reaction and the most common extractant is 2M KCl (Douglas and Bremner, 1970, Tabatabai and Bremner, 1970). Several methods have been used to detect the amount of urea hydrolyzed during an assay (1) measurement of residual urea in the soil (Douglas and Bremner, 1970, Keeney and Bremner, 1967, Simpson and Melsted, 1963), (2) determination of the ammonium produced by hydrolysis (Roberge, 1968), (3) the amount of $^{14}CO_2$ evolved from ^{14}C-labelled urea (Skujins and McLaren, 1969).

V. Urease Inhibitors

Most studies of urease inhibition have been with either jack bean or bacterial urease and the hydroxamic acids have received the most attention of any of the known urease inhibitors. The hydroxamic acids are specific and non-competitive inhibitors of urease (Gale and Atkins, 1969, Kobashi *et al*, 1962). There are differences in behavior between aliphatic and aromatic hydroxamic acids, the former are irreversible and inhibition is progressive with time and the latter are instantaneous reversible inhibitors (Kobashi *et al*, 1971). Inhibition of urease by hydroxamic acids is dependent on both the steric and hydrophobic properties of the substituent group (Kumaki *et al*, 1972). The most powerful inhibitor of the hydroxamate class is caprylohydroxamic acid (Hase and Kobashi, 1967).

Hydroxyurea and dihyroxyurea act as urease inhibitors but are also hydrolyzed. The hydrolysis curves are biphasic in nature, that is, an initial rapid rate of hydrolysis is followed by a slower hydrolysis rate (Fishbein, 1969b, Fishbein and Carbone, 1965, Gale, 1965). Hydroxylamine, one of the hydrolysis products for both hydroxyurea and dihydroxyurea is a non-competitive reversible inhibitor of urease (Fishbein, 1969b, Fishbein and Carbone, 1965).

It has been suggested that the catalytic and inhibitory sites are separate for hydroxyurea and dihyroxyurea (Fishbein, 1969b).

Oxytetracycline (Belding and Kern, 1963), suramin (Wills, 1952, Wills and Wormall, 1950), sulfite ions and bisulfite ions (Ambrose et al, 1950) have been shown to inhibit urease. Thiourea and methylurea are inhibitors of urease but the latter is a much stronger inhibitor than the former (Kistiakowsky and Shaw, 1953, Shaw and Raval, 1961a). Both of these compounds are non-competitive inhibitors above pH 7.0 but are competitive inhibitors at pH 6.0. Substituted phenylureas have also been shown to be inhibitors of jack bean urease (Cervelli et al, 1975).

Some metal ions inhibit urease and can be grouped in the following order of inhibitory power: $Ag^{+}>Hg^{++}>Cu^{++}>Cd^{++}>Mn^{++}\sim Co^{++}>Pb^{++}>Ni^{++}$ (Toren and Burger, 1968). The order of inhibition is inversely proportional to the Ksp values of the corresponding sulfides of the various metals (Hughes et al, 1969, Shaw, 1954). Thus, metals which form the most insoluble sulfides are the best inhibitors, which implies that metal ions inhibit urease by reacting with essential sulfhydryl groups at the active site (Shaw and Raval, 1961b).

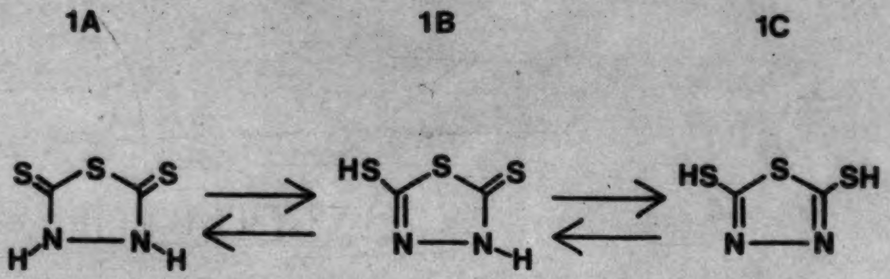
Dihydric phenols inhibit urease and the inhibition can be reversed by thiol compounds (Quastel, 1933). Quastel suggested that only the oxidized form of dihydric phenols, that is the corresponding quinones, are toxic to urease. Since thiols form addition products with some quinones (Cecil and McPhee, 1959), it is likely that dihydric phenols and quinones inhibit urease by blocking essential sulfhydryl groups at the active site.

Soil urease inhibitors have been investigated with a view to eliminating some of the problems caused by the rapid hydrolysis of fertilizer urea. Acetohydroxamic acid, dihydric phenols, and benzoquinones have been shown to be inhibitors of soil urease (Bremner and Douglas, 1971a, Bundy and Bremner, 1973). Dithiocarbamates have been patented as soil urease inhibitors (Hyson, 1963) but their effectiveness has been questioned by others (Bremner and Douglas, 1971a). Acetohydroxamic acid and p-chloromercuribenzoate reduce the maximum rate of ammonia volatilization from urea-fertilized soil, but the total amount of ammonia volatilized over an extended period of time is not changed by these inhibitors (Moe, 1967, Pugh and Waid, 1969a, 1969b). 2,5-Dimethyl-1,4-benzoquinone, a potent urease inhibitor has been found effective in reducing gaseous nitrogen loss from urea applied to a Thurman sand (Bremner and Douglas, 1973). Some urease inhibitors are also nitrification inhibitors which will result in ammonium accumulating over an extended period of time and the increased possibility of ammonia volatilization losses (Bundy and Bremner, 1974a, 1974b, Moe, 1967).

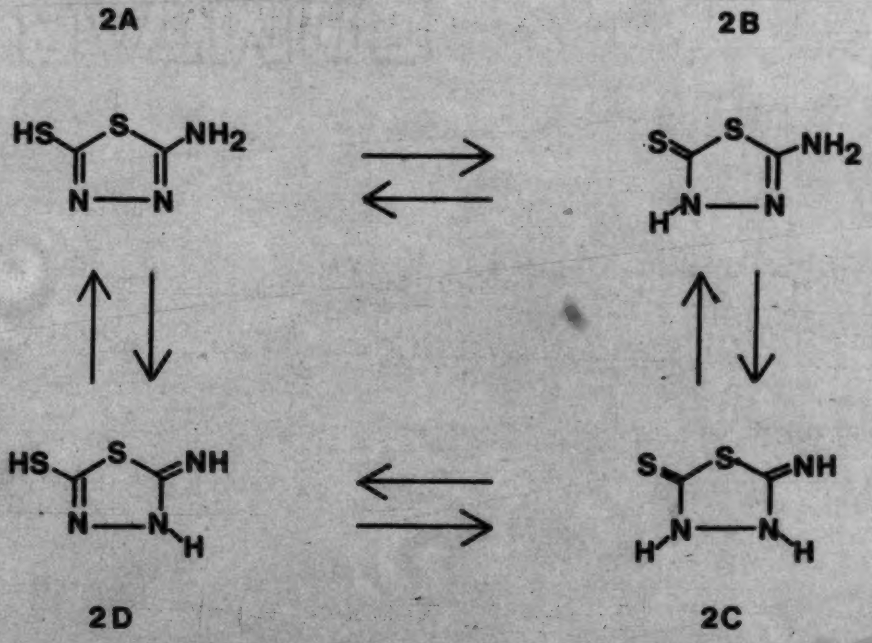
VI. Properties of the Mercapto-Substituted Thiadiazoles

Preliminary studies to evaluate a number of compounds for the inhibition of soil urease activity showed that several heterocyclic mercaptans were potent inhibitors of soil urease. Additional studies were carried out to elucidate the mechanism by which these compounds inhibit urease. The following heterocyclic mercaptans were studied in detail: 1,3,4-thiadiazole-2,5-dithiol, 5-amino-1,3,4-thiadiazole-2-thiol, 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione, and rhodanine

(2-thioxo-4-thiadiazolidinone). These compounds can also be named as the corresponding thione because the possibility of thione-thiol tautomerism exists. There are three tautomeric possibilities for 1,3,4-thiadiazole-2,5-dithiol (Sandstrom, 1968):



5-Amino-1,3,4-thiadiazole-2-thiol has four tautomeric possibilities (Sandstrom, 1968):



Similar tautomeric possibilities also exist for the other two compounds.

Although there is some dispute concerning the tautomeric form of heterocyclic thioamides, the thione form is probably the predominant one (Katritsky and Lagowsky, 1963, Sandstrom and Wennerbeck, 1966). Infrared spectroscopic studies have shown that 2-phenyl-1,3,4-thiadiazoline-5-thione probably exists as the thione tautomer in chloroform (Ainsworth, 1958). X-ray diffraction studies (Downie et al, 1972) to determine bond lengths indicate that in the solid state 5-amino-1,3,4-thiadiazole-2-thiol exists as the thione (2B). Thorn (1960) compared the ultraviolet spectra in 95% ethanol of 1,3,4-thiadiazole-2,5-dithiol with its S- and N- methyl derivatives and suggested that the dithione tautomer predominates (1A). However, in chloroform, infrared spectra show the presence of a thiol group (Thorn, 1960).

The α-effect, in which an electronegative atom such as nitrogen is adjacent to a nucleophile, such as sulfur, the reactivity of the nucleophile is enhanced (Jencks, 1969). The heterocyclic mercaptans considered in this study possess a nitrogen atom adjacent to the carbon atom with the mercapto substituent. Thus the mercapto group should be very reactive in either the thione or thiol tautomer.

1,3,4-Thiadiazole-2,5-dithiol forms a soluble, colored, 1:1 complex with palladium and also forms soluble complexes with several other divalent metal ions (Majumdar and Chakrabartty, 1958). The colored complexes formed by 1,3,4-thiadiazole-2,5-dithiol with palladium and bismuth have been used for the colorimetric determination of both of these elements (Majumdar and Chakrabartty, 1958).

VII. Summary

Urease is widely distributed in bacteria, yeasts and fungi, and is also found in some higher plants. Urease has been shown to have several different functions, but is primarily involved in nitrogen metabolism, particularly when nitrogen is limiting.

The mechanism of the urease-catalyzed hydrolysis of urea involves the enzymatic hydrolysis of urea to ammonium carbamate, followed by the chemical hydrolysis of the carbamate to ammonia and carbon dioxide, which at neutral pH is in equilibrium with ammonium bicarbonate. In some buffers the hydrolysis proceeds only as far as ammonium carbamate, and in others the complete hydrolysis takes place.

Values of 2 - 8 for the number of active sites per urease molecule have been reported. Urease has been shown to be a sulfhydryl dependent enzyme, and recent work indicates that urease is a nickel metalloenzyme.

Molecular weights of 483,000 and 487,000 have been reported for urease. Twelve active isozymes of urease have been reported. These include a half-unit of urease, several higher polymers, and a number of isozymes that differ only slightly from each other. Inactive structural subunits of molecular weights varying from 30,000 - 90,000 have also been found when urease is treated with dissociating agents. Variations in the proportions of various isozymes under different conditions are responsible for the anomalous kinetic behavior sometimes observed during the hydrolysis of urea by urease.

Urea is hydrolyzed very rapidly by soil urease to ammonium bicarbonate, and the ammonium ions can then be oxidized by the nitrifying bacteria in the soil to nitrite and then nitrate. Urease activity can be found in most soils, and the highest activities have been found in the surface horizons of forest soils. Most of the soil urease activity is extracellular and complexed with soil colloids and is stable for long period of time. Urea has occasionally been shown to be less efficient than other nitrogenous fertilizers. The problems with urea fertilizer are caused by toxicity to plants from nitrite and ammonia, and the loss of nitrogen by volatilization of ammonia.

The hydroxamic acids, hydroxyurea, dihydroxyurea, hydroxylmine, and the substituted ureas have been shown to be urease inhibitors. Heavy metals, some of the dihydric phenols, and the quinones all inhibit urease by reacting with the essential sulfhydryl groups of the enzyme.

The urease inhibitors of interest in this study are a number of heterocyclic mercaptans. These compounds can rearrange by a thione-thiol tautomerism, and in most studies the thione tautomer has been found to predominate. These mercapto groups are very reactive because of the close proximity of an electronegative nitrogen atom. 1,3,4-Thiadiazole-2,5-dithiol also forms colored complexes with several metal ions.

MATERIALS AND METHODS

I. MATERIALS

All chemicals used were of reagent grade. The urease was extracted from B grade jack bean meal, which was obtained from Caliochem, San Diego, California. Sepharose 2B and CH-Sepharose 4B were obtained from Pharmacia (Canada) Ltd., Dorval, Quebec. The protein standard, Bovine Serum Albumen (BSA), was obtained from Cyclo Chemical, Los Angeles, California. Acetohydroxamic acid, N'-methyl-N-hydroxyurea, crotonylidene diurea (CDU), formamidine acetate, acetamidine acetate, acetamidoxime, and crotonaldehyde thiourea were obtained from the Research Council of Alberta. Hydroxyurea, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide dihydrochloride, sym-dimethylhydrazine, carbon disulfide and all of the other compounds used for the inhibition studies were obtained from Aldrich Chemical Company Inc. 3,4-Dimethyl-2,5-dithione-1,3,4-thiadiazolidine, 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide, and 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide were synthesized as described in the methods section. The structures and names of all of the compounds considered as either jack bean urease or soil urease inhibitors are listed in Appendix I. The water used in the studies with jack bean urease was first de-ionized and then distilled in glass apparatus.

The ethanol (95%) used as solvent for the spectroscopic studies, and also as an inhibitor solvent was redistilled once by the following procedure. Approximately 5 litres of 95% ethanol was placed in a glass still, the first 500 ml of distillate was discarded, the subsequent 3 litres of distillate collected and the ethanol remaining in the still was discarded.

The soil sample used in this investigation was the Ap horizon of a Chernozem (Malmo silt loam) obtained from the University Farm at Ellerslie (NE24-51-25-W4). This location was seeded to grass during 1964-67 and was fallow from 1967-70. The plot (50' X 50') was randomly sampled in five locations and the five subsamples were mixed to produce a composite sample. The composite sample was air-dried for three days, ground to pass a 12 mesh sieve and stored in plastic bags at room temperature. The data from the mechanical and chemical analyses of the soil are listed in Appendix II.

II. METHODS

A. ROUTINE CHEMICAL ANALYSES

1. Protein:

Soluble protein was determined by the method of Lowry *et al*, (1951) using BSA as a standard. The relative protein content of column effluents was monitored by measuring the absorbance of the effluents at 280 nm on a Unicam SP 1800 Ultraviolet Spectrophotometer.

2. Soil Analyses:

The mechanical analysis of the soil sample was carried out by the pipette method (Kilmer and Alexander, 1949). Soil pH was determined in a 1:2.5 soil-water suspension (Peech, 1965). Cation exchange capacity was determined by leaching the sample with normal ammonium acetate, extracting the adsorbed ammonium with normal sodium chloride and distilling the extract by the magnesium oxide method (A.O.A.C., 1955). Total carbon content was determined by the Leco dry combustion method (McGill, 1976). The sample was placed in a

Leco induction furnace, oxidized, and the carbon dioxide evolved was measured manometrically.

3. Ammonium:

Ammonium ion concentrations were determined by the Fawcett and Scott (1960) version of the indophenol blue procedure. The sample was made up to a volume of 2 ml with deionized distilled water, and the following solutions were added immediately after each other to the sample: 2 ml of sodium phenate (25 g phenol and 12.5 g sodium hydroxide/liter), 3 ml of 0.01% sodium nitroprusside, and 3 ml of 0.02 N sodium hypochlorite solution. After 30 minutes at room temperature, the concentration of ammonium ions was calculated from the optical density of a colored complex measured at a wavelength of 630 nm (ϵ = 21,600).

The indophenol blue procedure involves the development of a blue dye by ammonia, hypochlorite, and phenol under alkaline conditions. Some variations of the indophenol blue procedure develop a color with urea. The specificity of this method is dependent on the order of addition of the reagents, and if the reaction is begun at low pH, a colored complex can be formed with urea as well as some amines and amino acids (Wearne, 1963). The method employed in this study is specific for only the ammonium ion.

4. Urea:

Urea was determined by the Watt and Chrisp (1954) method. The sample was made to a volume of 15 ml with distilled water and 10 ml of color reagent (10 ml concentrated HCl, 100 ml of 95% ethanol and 2.0 g of p-dimethylaminobenzaldehyde) were added. After 10 minutes at room temperature the urea concentration was calculated from the optical density of a colored complex measured at a wavelength of 430 nm ($\epsilon = 224$).

This method is less sensitive than some of the other methods used for the determination of urea but it was found to be reproducible and free of interferences.

B. PURIFICATION OF JACK BEAN UREASE

1. Recrystallization of Crude Jack Bean Meal:

Most of the experiments reported in this thesis were carried out with a once recrystallized preparation of jack bean meal. The meal was recrystallized by a modified version of the procedure of Mamiya and Gorin (1965). Jack bean meal (50 g) was suspended in the extraction solution (90 ml of acetone made up to 250 ml with water containing 1% (v/v)2-mercaptoethanol) at 39°C. After stirring for five minutes the mixture was filtered in a Buchner funnel and the filtrate stored at 4°C for 48 hours. The filtrate was then centrifuged at 37,000 X g for 15 minutes and the supernatant was discarded. The precipitate was suspended in 100 ml of 20 mM phosphate-1 mM EDTA buffer (pH 7.0). After 24 hours at 4°C the mixture was centrifuged at 37,000 X g for 15 minutes, and the supernatant was used for inhibition studies and further purification.

2. Preparation of the Affinity Gel:

Hydroxyurea was chosen as the affinity ligand since it has been successfully used by others for the purification of urease (Wong and Shobe, 1974). Three techniques were used to prepared affinity gels for urease: 1. Using the procedure outlined by Cuatrecasas and

Anfinsen (1971), Sepharose 2B was activated with cyanogen bromide, to which ethylenediamine was coupled, followed by succinylation with succinic anhydride, and the addition of hydroxyurea mediated by a water-soluble carbodiimide [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride]. This compound will be abbreviated as WSCD when referred to again in this thesis. 2. An affinity gel was also prepared by coupling hydroxyurea to CH-Sepharose 4B in the presence of the water-soluble carbodiimide (WSCD). 3. The procedure finally adopted involved the carbodiimide-mediated binding of ethylenediamine to CH-Sepharose 4B, followed by succinylation, and then the carbodiimide-mediated binding of hydroxyurea. The reaction sequence is outlined in Figure 1. The conditions described by Cuatrecasas and Anfinsen (1971) were used for each reaction. The following washing procedure was followed during the preparation of each one of the affinity gels. After each reaction was completed, the gel was transferred to a Buchner funnel and washed extensively before the next reaction was carried out. The gel was washed with three different wash solutions in the following sequence: 1.0 M NaCl (50 ml/ml of packed gel), 0.2 M KH_2PO_4 (50 ml/ml of packed gel) and distilled water (200 ml/ml of packed gel). The washing procedure was necessary to remove unreacted starting materials so they would not interfere with subsequent reactions. The presence or absence at each step of a free amino or hydroxylamino group was monitored using the 2,4,6-trinitrobenzene-sulfonate-sodium borate reagent (Cuatrecasas and Anfinsen, 1971). The prepared gel was washed (20 ml/ml of packed gel) with 20 mM phosphate-1 mM 2-mercaptoethanol buffer (pH 7.0) before it was used for the purification of urease.

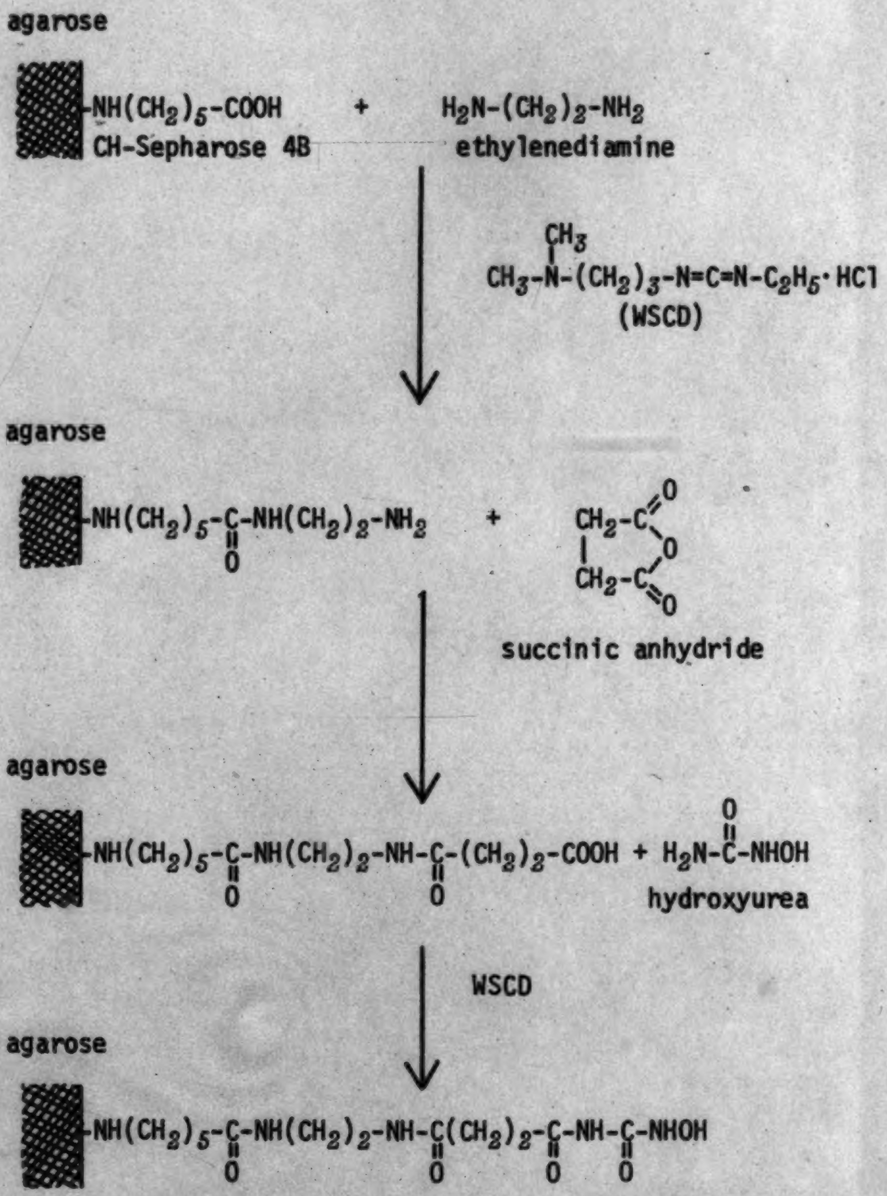

agarose
-NH(CH2)5-COOH
CH-Sepharose 4B
+
H2N-(CH2)2-NH2
ethylenediamine
CH3-N(CH3)-(CH2)3-N=C=N-C2H5·HCl
(WSCD)
agarose
-NH(CH2)5-C(=O)-NH(CH2)2-NH2
+
succinic anhydride
agarose
-NH(CH2)5-C(=O)-NH(CH2)2-NH-C(=O)-(CH2)2-COOH
+ H2N-C(=O)-NHOH
hydroxyurea
WSCD
agarose
-NH(CH2)5-C(=O)-NH(CH2)2-NH-C(=O)(CH2)2-C(=O)-NH-C(=O)-NHOH

Figure 1: Procedure for the preparation of the affinity gel that was used for the purification of jack bean urease (Procedure #3).

3. Purification of Urease by Affinity Chromatography:

The purification of jack bean urease was attempted using the three types of affinity gel that were described in the previous section. The procedure employed for the purification of urease by affinity chromatography was adopted from that employed by Wong and Shobe (1974). A column containing 50 ml of affinity gel was equilibrated with 20 mM phosphate-1 mM 2-mercaptoethanol buffer (pH 7.0). Approximately 15 to 30 ml of a partially purified jack bean urease solution was applied to the column. The column was then washed at a flow rate of 1.0 ml/minute with 20 mM phosphate-1 mM 2-mercaptoethanol buffer (pH 7.0) and fractions of 8-10 ml were collected. The column was washed until the absorbance of the effluent at 280 nm was less than 0.01. The elution buffer was then changed to 0.2 M phosphate-1 mM 2-mercaptoethanol buffer (pH 4.6) and the effluent fractions were monitored for urease activity. When urease activity could no longer be detected in the effluent, the gel was washed with 1.0 liter of 0.4 M phosphate buffer (pH 4.6) and 2.0 liters of distilled water and the gel was then equilibrated with 20 mM phosphate-1 mM 2-mercaptoethanol. If the affinity gel was to be stored for any length of time it was stored in 0.4 M phosphate buffer (pH 4.6). The fractions of the elution buffer containing urease activity were pooled and immediately dialyzed against four changes of 20 mM phosphate-1 mM EDTA buffer (pH 7.0) containing 1 mM 2-mercaptoethanol. The fifth change of buffer did not contain any 2-mercaptoethanol.

C. ENZYME ASSAYS

1. Jack Bean Urease:

Urease activity was assayed by the Chin and Gorin (1966) modification of Sumner's procedure (Sumner and Hand, 1928). This method is a fixed time assay and has the advantage of being relatively simple. The substrate contained 3.0 g of urea dissolved in sufficient 0.68 M phosphate-1 mM EDTA buffer (pH 7.0) to make 100 ml. The urease solution, which was stored at 4°C was diluted to the proper range for assay in 20 mM phosphate-1mM EDTA buffer (pH 7.0) and allowed to stand at room temperature for 2.0 hours. One ml of the urease solution was mixed with 1 ml of the substrate solution; after exactly 5 minutes at room temperature 1 ml of 1M H_2SO_4 was added quickly to stop the reaction. A 10-25 μl aliquot of the final reaction mixture was analyzed for the ammonium ion concentration by the previously described procedure.

The urease activity of column effluents was determined by taking 0.2 ml of each fraction, diluting to 1.0 ml with 20 mM phosphate-1 mM EDTA buffer (pH 7.0), and the urease activity was determined by the previously described procedure.

2. Soil Urease:

The urease activity of soil was determined by a method based on the one employed by Simpson and Melsted (1963). Unless otherwise stated, the soil urease experiments reported in this thesis were carried out under the following conditions. Each sample (25 g air-dry soil) was incubated in a 200 ml Erlenmeyer flask. Five milliliters of urea solution (8.6 g urea/liter) and 1.0 ml of distilled water were added to each sample to produce an initial substrate concentration of 400 ppm urea-N (w/w of soil), and a moisture concentration of 32% (w/w of soil, equivalent to field capacity). The substrate concentration was found to be in excess, and thus suitable for urease assay. Antimicrobial agents were not used in order to better simulate field conditions. The blank contained the same quantities of soil and water as the samples. The flasks were sealed with parafilm, placed in a constant temperature incubator, and they were incubated for 24 hours at 25°C. At the end of the incubation period the flasks were removed from the incubator, and the urea was extracted and analyzed.

In order to extract urea from the soil, the following were added to each flask: 0.2 g $CaCl_2$, 0.2 g Norit-A decolorizing carbon, 5 ml of 1% $HgCl_2$ solution, and enough distilled water to dilute to a total volume of 100 ml. The samples were shaken for 20 minutes and filtered through Whatman #1 filter paper. Ten milliliters of urea color reagent were added to 15 ml of the filtrate, and the urea concentration was determined by the previously described procedure. The amount of urea that had been hydrolyzed was calculated by difference.

The purpose of the mercuric chloride in the extracting solution was to stop the reaction. Calcium chloride was used for two reasons; (1) calcium causes the clay to floculate during extraction and thereby produces a clear filtrate; (2) calcium chloride is a neutral salt, and the color produced by the Watt and Chrisp (1954) procedure is pH sensitive.

D. INHIBITION STUDIES

1. Jack Bean Urease:

A once recrystallized jack bean urease preparation (Specific Activity = 5-10 S.U./mg of protein) was used in these studies. Prior to the inhibition studies, the urease was dialyzed against 20 mM phosphate-1 mM EDTA buffer (pH 7.0) for three days at 4°C (20 ml enzyme was dialyzed against 100 ml buffer). The dialysis buffer was changed every 24 hours. The dialysis procedure was necessary to remove trace amounts of 2-mercaptoethanol which interfered with some of the inhibitors.

From 2 to 50 µl of inhibitor solution were added to 0.2 Sumner Units (S.U.) of urease dissolved in 1.0 ml of 20 mM phosphate-1 mM EDTA buffer (pH 7.0), the mixture was preincubated for 4.0 hours, followed by the addition of substrate and determination of enzyme activity as previously described. The preincubation time of 4.0 hours was necessary to allow the urease and the inhibitor to come to equilibrium (see Results and Discussion, Time Studies).

The following solvent systems were used to dissolve the inhibitors; acetone, ethanol, redistilled ethanol, water and ethanol containing 0.5% DMSO (v/v). The heterocyclic mercaptans readily dissolved in ethanol, but it was necessary to add an equimolar amount of sodium hydroxide to dissolve them in water. Control experiments were carried out using only the inhibitor solvents, and the solvents were found to have no effect on urease activity. Each preparation of inhibitor was arbitrarily assigned a preparation number in order to distinguish between the various treatments.

Inhibitory action was calculated as % inhibition:

$$\% \text{ inhibition} = 100 - \left[\frac{\text{mg } NH_4^+\text{-N produced by enzyme + inhibitor}}{\text{mg } NH_4^+\text{-N produced by enzyme}} \times 100 \right]$$

The index of inhibition (I_{50}) was taken as the concentration of inhibitor in the reaction volume (2.0 ml) necessary to produce a 50% inhibition of urease.

The compounds that were studied were: 1,3,4-thiadiazole-2,5-dithiol; 5-amino-1,3,4-thiadiazole-2-thiol; 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione; rhodanine; 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide; 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide; acetohydroxamic acid; and 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene. 5-Mercapto-3-phenyl-1,3,4-thiadiazole-2-thione was converted to the acid by dissolving the compound in water and precipitating it with hydrochloric acid.

2. Soil Urease:

Unless otherwise specified, the soil urease inhibition studies were carried out using the assay conditions previously described. Each soil sample used for these studies was treated with the urea solution that also contained the inhibitor at a concentration equivalent to 100 ppm (w/w) of soil. The inhibitors that were insoluble in water were added as a slurry. The inhibitor concentration of 100 ppm (w/w of soil) falls within the range of values (50-300 ppm, w/w of soil) employed in previously published work (Bremner and Douglas, 1971a). The inhibitors were omitted from the control samples.

The blank, the control samples, and the samples that contained the inhibitors, were incubated, extracted and the urea analyzed by the previously described procedure.

The % inhibition was calculated in the following manner:

$$\% \text{ inhibition} = 100 - \left[\frac{\text{initial urea concentration} - \text{final urea concentration with inhibitor}}{\text{initial urea concentration} - \text{final urea concentration in control samples}} \times 100 \right]$$

All of the calculated results are the means of triplicate determinations.

Each of the compounds that was assayed for inhibitory power, was added to soil, the incubation and extraction procedure was carried out, and the filtrate tested for interference with the urea color reagent. None of the compounds interfered with the color reagent.

3. Effect of EDTA on the Inhibition of Jack Bean Urease by 1,3,4-Thiadiazole-2,5-dithiol:

EDTA (disodium salt) was added to an ethanolic solution of 1,3,4-thiadiazole-2,5-dithiol as a means of determining the role of metal-thiazole complexes in the inhibition of urease. Sodium hydroxide was added to solubilize the EDTA. The solution contained the following concentrations of each compound: 1,3,4-thiadiazole-2,5-dithiol (5.0×10^{-3}M), EDTA (2.5×10^{-3}M), and sodium hydroxide (2.5×10^{-3}M). An experiment was also carried out using identical concentrations of 1,3,4-thiadiazole-2,5-dithiol and sodium hydroxide but without EDTA. These ethanolic solutions were tested for inhibitory power against jack bean urease by the previously described procedure.

4. Inhibition of Jack Bean Urease by Metal Complexes of 1,3,4-Thiadiazole-2,5-dithiol:

Metal ions were added to solutions of 1,3,4-thiadiazole-2,5-dithiol in order to determine if the metal complexes of these compounds were inhibitory. The various metal salts were each dissolved in water and a small amount of these solutions (<1% of final volume) were added to ethanolic solutions of 1,3,4-thiadiazole-2,5-dithiol. The final concentration of 1,3,4-thiadiazole-2,5-dithiol was 10^{-3}M and the molar ratios of the thiadiazole to the various metal are given in the Results and Discussion section. Within two hours of preparing these solutions they were added to jack bean urease for inhibition studies.

Solutions of a number of metal salts were also added to ethanolic and alkaline aqueous (0.1 N NaOH) solutions of 1,3,4-thiadiazole-2,5-dithiol in order to determine which of the metals formed soluble colored complexes with the thiadiazole.

5. Pretreatment of Heterocyclic Mercaptans with Hydrogen Peroxide:

Three of the compounds considered in this study were treated with hydrogen peroxide to determine if the oxidized forms of the heterocyclic mercaptans were inhibitory. The following procedures were used: (1) An ethanolic solution was made up with the following concentrations of mercaptan and oxidant: 5-amino-1,3,4-thiadiazole-2-thiol (4.0 X 10^{-3}M) and hydrogen peroxide (4.0 X 10^{-3}M); (2) An ethanolic solution was made up containing rhodanine (10^{-3}M) and hydrogen peroxide (10^{-3}M). (3) An ethanolic solution was made up containing 1,3,4-thiadiazole-2,5-dithiol (10^{-3}M) and hydrogen peroxide

(10^{-3}M). All three of these solutions were allowed to stand at room temperature for 24 hours and then stored at 4°C.

The solutions containing the oxidized compounds were then tested for inhibitory power using the previously described technique. Control experiments using the same solutions without the hetercyclic mercaptans were also carried out and no effect on urease activity was found.

6. Effect of Reducing Agents on the Inhibition of Jack Bean Urease by Heterocyclic Mercaptans:

Two enzyme preparations [0.2 S.U./ml, dissolved in 20 mM phosphate-1 mM EDTA buffer (pH 7.0)] were treated with sodium sulfite and 2-mercaptoethanol in order to assess the effect of reducing agents on the inhibition of jack bean urease by the heterocyclic mercaptans. One enzyme preparation was treated with sufficient sodium sulfite solution (10^{-2}M) to produce a final concentration of 1mM, and the other enzyme solution was treated with sufficient 2-mercaptoethanol to produce a final concentration of 1 mM. Both of the enzyme solutions were preincubated for 1 hour at room temperature, and then each preparation was used for a series of inhibition studies employing four different inhibitors: 1,3,4-thiadiazole-2,5-dithiol; 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione; rhodanine (oxidized with hydrogen peroxide as previously described); 5-amino-1,3,4-thiadiazole-2-thiol (oxidized with hydrogen peroxide as previously described). The inhibition studies were carried out by the previously described procedure.

7. Dialysis Studies:

Four preparations of jack bean urease were each inhibited by the addition of an excess of one of the heterocyclic mercaptans and then each one was dialyzed against different buffer systems in order to determine what conditions were capable of reversing the inhibition. Urease solutions of activity 0.2 S.U./ml dissolved in 20 mM phosphate-1 mM EDTA buffer (pH 7.0) were used. Ethanolic solutions of each inhibitor were added to different urease preparations to produce the following concentrations in the enzyme solutions: 1,3,4-thiadiazole-2,5-dithiol, 1.3 X 10^{-4}M; 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione, 8.0 X 10^{-4}M; 5-amino-1,3,4-thiadiazole-2-thiol, 2.0 X 10^{-4}M (oxidized with hydrogen peroxide as previously described); and rhodanine, 6.0 X 10^{-5}M (oxidized with hydrogen peroxide as previously described). Each enzyme solution with its respective inhibitor was allowed to incubate at room temperature for 4.0 hours, and then assayed for urease activity. Three of the urease solutions were inactive, but the urease solution that had been treated with rhodanine still had some residual activity.

Each inhibited enzyme solution was then divided into three fractions and each fraction was dialyzed at 4°C against a different buffer system (25 ml enzyme was dialyzed against 100 ml buffer). The three buffer systems were: (1) 20 mM phosphate-1 mM EDTA (pH 7.0) (2) 20 mM phosphate-1 mM 2-mercaptoethanol-1 mM EDTA buffer (pH 7.0) (3) 20 mM phosphate-1 mM sodium sulfite-1 mM EDTA buffer (pH 7.0). A control sample, consisting of uninhibited urease was also dialyzed against 20 mM phosphate-1 mM EDTA buffer (pH 7.0). The activity of the control sample did not change appreciably during the course of the experiment.

The dialysis buffers were changed every 12 hours, and the urease activity was determined in duplicate every 24 hours for the first three days. The dialysis buffers were changed twice more over a period of two days and on the fifth day the urease activity was determined again. All of the enzyme solutions were then dialyzed for 24 hours against three changes of 20 mM phosphate-1 mM EDTA buffer (pH 7.0), and the final urease activity was then determined. The activity was expressed as % original activity:

$$\%\ \text{original activity} = \frac{\text{activity of inhibited sample (in S.U./ml)}}{\text{activity of control (in S.U./ml)}} \times 100$$

8. Time Studies:

A series of experiments were carried out to determine the rates at which a number of inhibitors inactivated jack bean urease.

A urease preparation [0.2 S.U./ml, dissolved in 20 mM phosphate-1 mM EDTA buffer (pH 7.0)] was allowed to stand at room temperature for 2.0 hours. An ethanolic solution of the inhibitor was then added to the enzyme preparation. At a number of subsequent time intervals, two aliquots of 1.0 ml each were removed from the enzyme-inhibitor reaction mixture and assayed for urease activity by the previously described technique. The % inhibition was then calculated for each sample time. This procedure was repeated for each inhibitor. The following inhibitors were employed in this study: 1,3,4-thiadiazole-2,5-dithiol; rhodanine (oxidized with hydrogen perioxide as previously described); 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide; and 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide. The inhibitor concentrations and preparation numbers are listed in the appropriate section of the Results and Discussion. The latter two compounds just previously listed are the corresponding dimers of two of the heterocyclic mercaptans that were studied. The dimers were chosen for the time studies because the inhibitory activity of each mercaptan was actually caused by trace amounts of the corresponding disulfide (dimer) in the mercaptan preparation. This topic will be discussed more fully in the Results and Discussion section.

E. SYNTHETIC CHEMISTRY

1. Procedure for the Preparation of 3,4-Dimethyl-2,5-dithione-1,3,4-thiadiazolidene:

Since this compound is not commercially available it had to be synthesized. Stoichiometric amounts of sym-dimethylhydrazine dihydrochloride, potassium hydroxide and carbon disulfide were refluxed in ethanol for 9 hours. The compound was precipitated by adding water and purified by recrystallizing once from 95% ethanol (Thorn, 1960). The structure was verified by infrared, n.m.r., ultraviolet, and mass spectroscopy. The spectroscopic experiments will be discussed in a later section.

2. Preparation of Dimerized Thiadiazoles:

Dimers of two of the heterocyclic mercaptans were prepared by oxidation with an excess of hydrogen peroxide. A solution of 30% hydrogen peroxide (0.15 moles) was added with rapid stirring to 0.1 moles (13.3 g) of 5-amino-1,3,4-thiadiazole-2-thiol dissolved in 1,000 ml of 95% ethanol. The solution was allowed to stand at room temperature for 24 hours, the precipitate was filtered, washed with 500 ml of 95% ethanol, and dried under vacuum for 48 hours. The product, 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide, was then used for inhibition studies.

A solution of 30% hydrogen peroxide (0.0037 moles) was added with rapid stirring to 0.0025 moles (0.565 g) of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione dissolved in 300 ml of 95% ethanol. The solution was allowed to stand at room temperature for 24 hours, the precipitate was filtered, washed with 200 ml of 95% ethanol, and dried under vacuum for 48 hours. The product, 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide was then used for inhibition studies.

The monomeric and dimeric forms of both of these heterocyclic mercaptans were studied by infrared, n.m.r., ultraviolet, and mass spectroscopy. A small quantity of each dimer was purified by sublimation under vacuum at 200°C for 24 hours, and then they were both used for additional mass spectroscopic studies.

F. SPECTROSCOPY

The spectroscopic studies of 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene were carried out using the following instruments. A Beckman Model IR-20 grating Infrared Spectrophotometer was used to obtain the infrared spectrum, a Varian Associates Model A50/60 Spectrometer was used to obtain the Nuclear Magnetic Resonance (n.m.r.) spectrum, a Unicam SP1800 Ultraviolet Spectrometer was used to obtain the ultraviolet spectrum, and an AEI Model MS-2 Mass Spectrometer was used to obtain the mass spectrum. The infrared spectrum was obtained using sodium chloride cells, and the sample was dissolved in chloroform. The sample was dissolved in deuterated chloroform ($CDCl_3$) in order to obtain the n.m.r. spectrum, and the sample was dissolved in redistilled 95% ethanol for the ultraviolet study.

The spectroscopic studies of 5-amino-1,3,4-thiadiazole-2-thiol, 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione, 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide and 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide were carried out using the following instruments. A Perkin-Elmer Model 451 grating Infrared Spectrophotometer was used to obtain infrared spectra, a Varian Associates Model 100 Spectrometer was used to obtain the n.m.r. spectra, a Unicam SP 1800 Ultraviolet Spectrometer was used to obtain the ultraviolet spectra, and an AEI Model MS-2 Mass Spectrometer was used to obtain the mass spectra. The samples were made up in potassium bromide (KBr) pellets in order to obtain the infrared spectra, and the samples were dissolved in deuterated dimethyl sulfoxide ($DMSO_{d6}$) to obtain the n.m.r. spectra. The sample solvent used in the ultraviolet studies was redistilled 95% ethanol.

The ultraviolet molar extinction coefficients were calculated from an average of three determinations.

RESULTS AND DISCUSSION

I. Purification of Jack Bean Urease

The recrystallization of urease from jack bean meal in an acetone-water mixture gave a low yield (Table 1), but the preliminary purification was necessary to produce a suitable preparation for affinity chromatography because the crude solutions gave poor results. The chemical structures of the three types of gel that were used in the various attempts to purify jack bean urease are listed in Figure 2. The urease solutions that had been chromatographed on gels prepared by procedures 1 and 2 were of very low specific activity. However, CH-Sepharose 4B, to which ethylenediamine, succinic anhydride, and hydroxyurea were bound (procedure 3) was successful (Figure 3, Table 1). The length of the side chain between the agarose matrix and the hydroxyurea ligand is 8 units for the gel prepared by procedure 1, and 7 and 15 units for procedures 2 and 3 respectively (Figure 2). The longer side chain obtained by binding the additional compounds in procedure 3 is necessary for purification of urease to be successful. Steric hinderance in the binding of proteins to affinity columns has been shown to be important for urease (Shobe and Brosseau, 1974), and other enzymes as well (Cuatrecasas and Anfinsen, 1971). Most of the inhibition studies were carried out using the preparation obtained from the acetone-water recrystallization. For comparison, one study was carried out with jack bean urease that had been purified by affinity chromatography.

The specific activity of the jack bean urease purified by affinity chromatography is within the range of values that have been

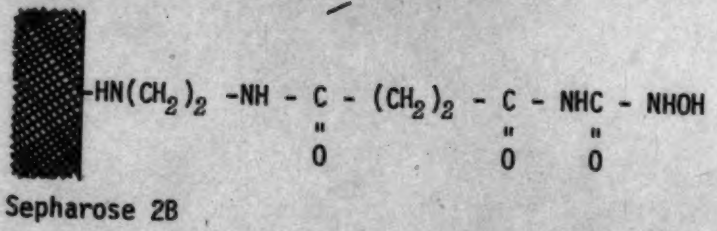
Table 1.

Purification of jack bean urease by recrystallization from an acetone-water mixture, followed by affinity chromatography.

Procedure	Volume, ml	Concentration, S.U./ml	Total Activity, S.U.	Protein, mg/ml	Specific Activity, S.U./mg	% Yield	Purification Factor
	150 [a]	16.0 [a]	2400	24.8 [a]	0.645	100	--
recrystallization from an acetone-water mixture	15	1.8	27.0	0.210	8.5	1.1	13.2
affinity chromatography	17	0.92	15.6	0.0088	105	0.65	164

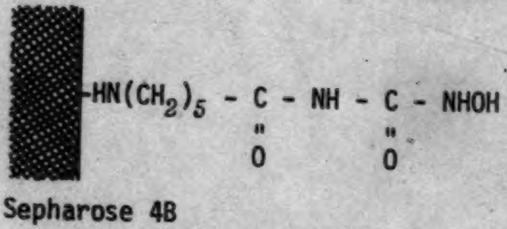
[a] Corrected values. The acetone water recrystallization was carried out using crude jack bean meal. The volumes and concentrations are based on a preliminary assay of 2.5 g of jack bean meal dissolved in 50 ml of 20 mM phosphate-1 mM EDTA buffer (pH 7.0). (Activity of crude meal: 320 S.U./g).

Procedure 1



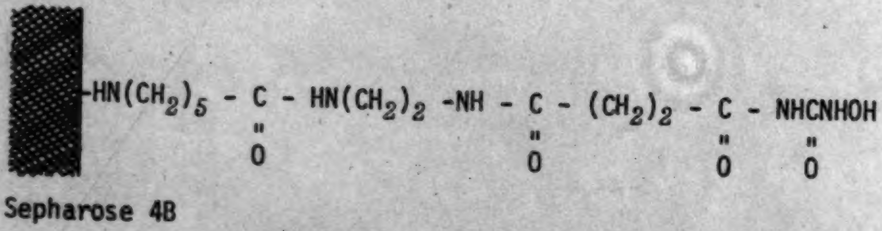
$$-\mathrm{HN(CH_2)_2-NH-\underset{\underset{O}{\|}}{C}-(CH_2)_2-\underset{\underset{O}{\|}}{C}-NH\underset{\underset{O}{\|}}{C}-NHOH}$$

Procedure 2



$$-\mathrm{HN(CH_2)_5-\underset{\underset{O}{\|}}{C}-NH-\underset{\underset{O}{\|}}{C}-NHOH}$$

Procedure 3



$$-\mathrm{HN(CH_2)_5-\underset{\underset{O}{\|}}{C}-HN(CH_2)_2-NH-\underset{\underset{O}{\|}}{C}-(CH_2)_2-\underset{\underset{O}{\|}}{C}-NH\underset{\underset{O}{\|}}{C}NHOH}$$

Figure 2: Side chain structures of the three types of affinity gels prepared for the purification of jack bean urease by affinity chromatography.

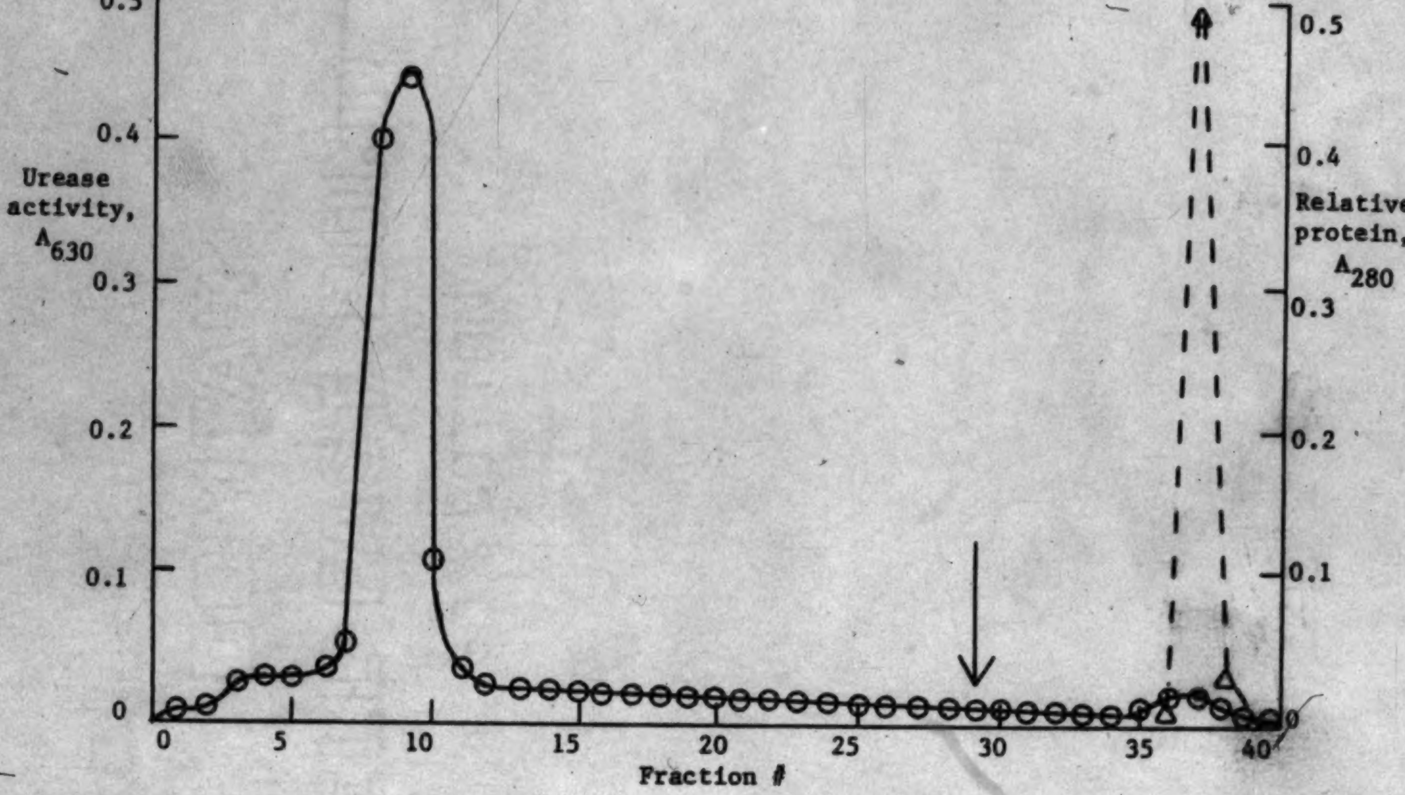


Figure 3: Purification of jack bean urease by affinity chromatography. A column containing 50 ml (packed volume) of gel prepared by procedure #3 was employed. Urease of specific activity 8.5 S.U./mg protein was added to the column in 20mMphosphate-1mM 2-mercaptoethanol buffer(pH7.0) and was chromatographed at a flow rate of 1 ml/min.(8.5 ml/fraction). After 29 fractions were collected (arrow) the elution buffer was changed to 0.2M phosphate(pH4.6). Fractions 37 and 38 were pooled and dialyzed. Urease activity, A_{630} △ —△— △ Relative protein, A_{280} ⊖—⊖—⊖

reported in the literature (Table 1). The specific activity of pure urease preparations has generally ranged from 80-170 S.U./mg protein (Gorin et al, 1962, Hanabusa, 1961, Hill and Elliott, 1966, Shadaksharaswamy and Hill, 1962), although values as high as 500 S.U./mg protein have been reported (Lynn, 1971b).

II. Inhibition of Jack Bean Urease

A. Preliminary Studies

The inhibition of jack bean urease by 1,3,4-thiadiazole-2,5-dithiol is illustrated by Figure 4. This type of inhibition curve is typical for most of the heterocyclic mercaptans discussed in this thesis. The concentration at which inhibition occurs is quite abrupt. The inhibition of jack bean urease by both 1,3,4-thiadiazole-2,5-dithiol and 5-amino-1,3,4-thiadiazole-2-thiol is affected by the solvent in which the inhibitor is initially dissolved (Tables 2 and 3). There is a tenfold increase in the inhibitory power of these compounds when ethanol rather than water is used as the inhibitor solvent (Tables 2 and 3). There is significant variation in the results, and even using the same inhibitor solvent, ethanol, the I_{50} values varied from 1.4 to 6.4 x 10^{-5} M for different preparations of 1,3,4-thiadiazole-2,5-dithiol (Table 2). The same type of experimental variation was also observed for 5-amino-1,3,4-thiadiazole-2-thiol (Table 3). However, a number of different experiments with the same preparation of 1,3,4-thiadiazole-2,5-dithiol (preparation 8A) gave reproducible I_{50} values (Table 2). Inhibition was also unaffected by enzyme purity (Table 2). Thus, the once recrystallized preparation of jack bean urease was considered to be satisfactory for inhibition studies.

5-Mercapto-3-phenyl-1,3,4-thiadiazole-2-thione is intermediate in potency when compared as a urease inhibitor with the other two heterocyclic mercaptans (Table 3). Ethanolic solutions of 1,3,4-thiadiazole-2,5-dithiol and 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione are an intense yellow color. The intensity of the color of

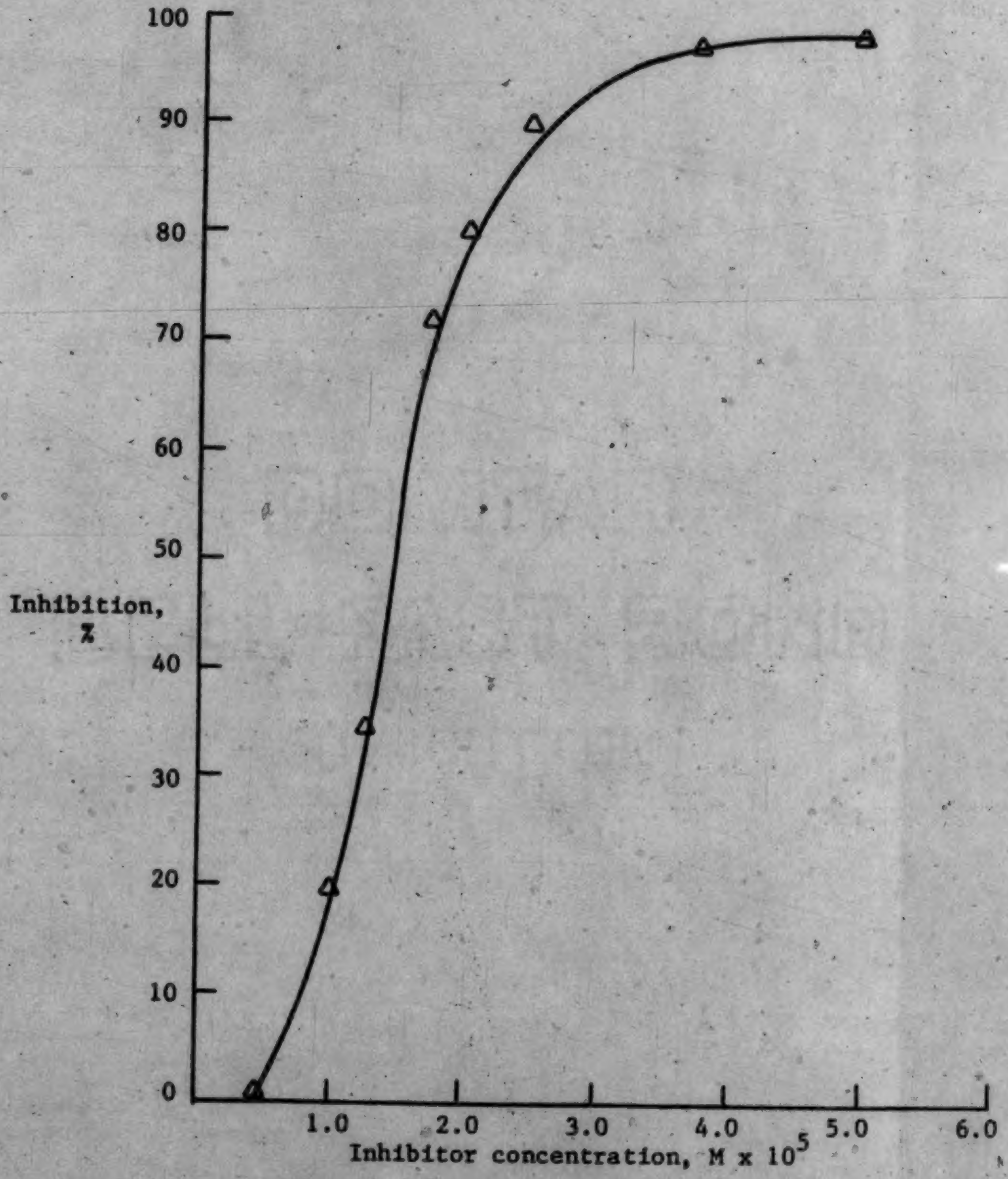


Figure 4: Inhibition of jack bean urease by 1,3,4-thiadiazole-2,5-dithiol. Inhibitor preparation #5A. ($I_{50} = 1.55 \times 10^{-5}$M)

Table 2

Inhibition of jack bean urease by 1,3,4-thiadiazole-2,5-dithiol. Experimental variation and effect of the inhibitor solvent.

Preparation Number	Inhibitor Solvent	I_{50}, M
1A	water	1.0×10^{-3}
2A	water	7.8×10^{-4}
3A	ethanol	2.6×10^{-5}
4A	ethanol	2.5×10^{-5}
5A	ethanol	1.6×10^{-5}
6A	ethanol	1.4×10^{-5}
7A	ethanol-redistilled	6.4×10^{-5}
8A	ethanol-redistilled	1.8×10^{-5}
8A	ethanol-redistilled	1.8×10^{-5}
8A [a]	ethanol-redistilled	1.8×10^{-5}

[a] Urease of 105 S.U./mg used.

Table 3

Inhibition of jack bean urease by 5-amino-1,3,4-thiadiazole-2-thiol, 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione, 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene, and the effect of the inhibitor solvent.

Inhibitor	Inhibitor preparation number	Inhibitor solvent	I_{50}, M
5-Amino-1,3,4-thiadiazole-2-thiol	1B	water	none [a]
5-Amino-1,3,4-thiadiazole-2-thiol	2B	water	2.3×10^{-3}
5-Amino-1,3,4-thiadiazole-2-thiol	3B	ethanol	9.6×10^{-4}
5-Amino-1,3,4-thiadiazole-2-thiol	4B	ethanol-redistilled	none [b]
5-Amino-1,3,4-thiadiazole-2-thiol	5B	ethanol-redistilled	6.7×10^{-4}
5-Mercapto-3-phenyl-1,3,4-thiadiazole-2-thione	1C	ethanol-redistilled	3.4×10^{-4}
3,4-Dimethyl-2,5-dithione-1,3,4-thiadiazolidene	1D	ethanol-redistilled	none [c]
3,4-Dimethyl-2,5-dithione-1,3,4-thiadiazolidene	2D	acetone	none [d]

a. No inhibition observed at inhibitor concentrations up to 3.5×10^{-3} M.

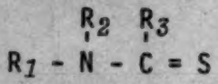
b. No inhibition observed at inhibitor concentrations up to 1.25×10^{-3} M.

c. No inhibition observed at inhibitor concentrations up to 1.25×10^{-4} M.

d. No inhibition observed at inhibitor concentrations up to 2.5×10^{-3} M. When added to the enzyme solution this compound precipitated at final concentrations above 10^{-3} M.

these preparations was observed to be proportional to their inhibitory power.

In Table 3, for example, one of the preparations of 5-amino-1,3,4-thiadiazole-2-thiol had no effect on jack bean urease even at the highest inhibitor concentration, and thus no I_{50} was given. The maximum concentration of that compound that was employed is listed in a footnote to the table. That format for noting the non-inhibitory preparations is used in all of the subsequent tables.

In order to explain the experimental variation and the inhibitor solvent effects, it was necessary to propose and investigate a number of different possible inhibition mechanisms during this study. Neutral amino thiones possessing the  $R_1 - \overset{R_2}{N} - \overset{R_3}{C} = S$ moiety are strongly nucleophilic due to the presence of an electronegative nitrogen atom (Carlsson et al, 1974a), which is also observed with hydroxylamine, hydroxamic acids, oximes and a variety of other compounds. Hydroxylamine and hydroxamic acids are good urease inhibitors, so it is possible that heterocyclic mercaptans inhibit urease when they tautomerize to the thione form. 1,3,4-Thiadiazole-2,5-dithiol was most inhibitory when it was dissolved in ethanol (Table 2), and the dithione tautomer is considered to be the dominant form in ethanol (Thorn, 1960). However, 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene, which is forced into the dithione tautomer by having a methyl group substituted on each nitrogen, was found to be ineffective (Table 3). Thus, it is unlikely that the inhibition of urease by the heterocyclic mercaptans can be attributed to the thione tautomers of these compounds.

B. Metal Complex Studies

Ethanolic solutions of 1,3,4-thiadiazole-2,5-dithiol and 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione were a yellow color, and the inhibitory power of each preparation was observed to be proportional to the intensity of the color. These compounds form stable colored complexes with a number of metal ions. The inhibitory species might be a metal-ligand complex formed by the reaction of the thiadiazole with trace amounts of metal ions present as impurities in either the compounds themselves or the reagents that were used.

The metal complexes of 1,3,4-thiadiazole-2,5-dithiol were the only ones studied in this investigation. The addition of EDTA and sodium hydroxide to an ethanolic solution of 1,3,4-thiadiazole-2,5-dithiol produced a preparation that was colorless as well as non-inhibitory (Table 4). The sodium hydroxide was added to solubilize the EDTA. The removal of the inhibitory power of 1,3,4-thiadiazole-2,5-dithiol by EDTA implies that inhibition is mediated by a metal ion. However, the addition of only sodium hydroxide also eliminated both color and the inhibitory power of the preparation (Table 4). When distilled water was used as an inhibitor solvent in the preliminary studies (Tables 2 and 3), sodium hydroxide was used to dissolve the inhibitors. Thus, the decreased potency of the heterocyclic mercaptans when they were initially dissolved in water was likely caused by the sodium hydroxide that had been added.

None of the metals that were considered in this study formed soluble colored complexes with 1,3,4-thiadiazole-2,5-dithiol

Table 4

Inhibition of jack bean urease activity by metal complexes of 1,3,4-thiadiazole-2,5-dithiol and the effects of EDTA and sodium hydroxide on the inhibition of urease by 1,3,4-thiadiazole-2,5-dithiol. Inhibitor solvent redistilled ethanol.

Inhibitor preparation number	Composition of inhibitor preparation	I_{50}, M
8A	inhibitor (I)/Co^{++} = 100	1.8 X 10^{-5} [a]
8A	(I)/Fe^{++} = 100	1.9 X 10^{-5}
8A	(I)/Zn^{++} = 100	1.8 X 10^{-5}
8A	(I)/Sn^{++} = 100	1.6 X 10^{-5}
8A	(I)/Sn^{++} = 10	1.4 X 10^{-5}
9A	---	3.0 X 10^{-5}
9A	(I)/Ni^{++} = 100	3.0 X 10^{-5}
9A	(I)/Cu^{++} = 100	3.1 X 10^{-5}
9A	(I) + EDTA + NaOH	none [b]
9A	(I) + NaOH	none [b]

[a] I_{50} value for the control is given for preparation 8A in Table 2.

[b] No inhibition observed at inhibition concentrations up to 1.25 X 10^{-4} M.

Table 5

Formation of soluble colored complexes between various metal ions and 1,3,4-thiadiazole-2,5-dithiol.

		Ethanol		Water	
Ion	Salt Used	Solubility	Supernatant color	Solubility	Supernatant color
Fe^{++}	$FeSO_4$	trace	none	trace	none
Fe^{+++}	Fe citrate	soluble	none	soluble	none
Mn^{++}	$MnSO_4$	trace	none	trace	none
Sn^{++}	$SnCl_2$	very soluble	orange	very soluble	none
Ni^{++}	$NiCl_2$	soluble	none	trace	none
Cu^{++}	$CuSO_4$	trace	none	trace	none
Mg^{++}	$MgSO_4$	soluble	none	trace	none
Ca^{++}	$CaCl_2$	soluble	none	trace	none
Pb^{++}	Pb acetate	trace	none	trace	none
Al^{+++}	$AlK(SO_4)_2$	trace	none	soluble	none
Zn^{++}	$ZnSO_4$	soluble	none	trace	none
Ba^{++}	$Ba(OH)_2$	trace	none	trace	none
Co^{++}	$CoCl_2$	trace	none	trace	none
Cd^{++}	Cd acetate	trace	none	trace	none
Ag^{+}	$AgNO_3$	trace	none	trace	none

in alkaline aqueous solutions, and only the stannous ion formed a soluble colored complex with 1,3,4-thiadiazole-2,5-dithiol in ethanolic solutions (Table 5). No change in the inhibitory power could be observed when various metal ions were added to ethanolic solutions of 1,3,4-thiadiazole-2,5-dithiol (Table 4). The stannous ion, which formed a soluble colored complex with 1,3,4-thiadiazole-2,5-dithiol, only caused a slight increase in the inhibitory power of the inhibitor solution (Table 4). Thus, it is unlikely that the inhibition of jack bean urease by the heterocyclic mercaptans can be explained by the interaction of a metal-thiazole complex with the enzyme.

Recent work suggests that urease is a nickel metalloenzyme (Dixon _et al_, 1975). Inhibition of urease could occur via a reaction between a heterocyclic mercaptan and a nickel atom of the urease molecule. This particular hypothesis was not explored because another proposed mechanism was found to be satisfactory.

The role of interactions between the mercapto group of the heterocyclic mercaptan and the sulfhydryl (mercapto) groups of urease will be discussed in subsequent sections of this thesis.

C. Effect of Oxidized Heterocyclic Mercaptans on Urease

Ethanolic solutions of three of the heterocyclic mercaptans were oxidized with hydrogen peroxide. The ethanolic solutions of 5-amino-1,3,4-thiadiazole-2-thiol and rhodanine were not inhibitory prior to peroxide treatment but after they had been oxidized were potent urease inhibitors (Table 6). When hydrogen peroxide was added to an ethanolic solution of 1,3,4-thiadiazole-2,5-dithiol, a

Table 6

Inhibition of jack bean urease by oxidized heterocyclic mercaptans. Inhibitor solvent redistilled ethanol.

Inhibitor	Inhibitor preparation number	Composition of inhibitor preparation	I_{50}, M
rhodanine	1E	thiazole	none [a]
rhodanine	1E	thiazole/H_2O_2 = 1	1.9×10^{-5}
1,3,4-thiadiazole-2,5-dithiol	10A	thiazole	2.2×10^{-5}
1,3,4-thiadiazole-2,5-dithiol	10A	thiadiazole/H_2O_2 = 1	1.9×10^{-5}
5-amino-1,3,4-thiadiazole-2-thiol	6B	thiazole	none [b]
5-amino-1,3,4-thiadiazole-2-thiol	6B	thiadiazole/H_2O_2 = 1	5.2×10^{-5}

a. No inhibition observed at inhibitor concentrations up to 2.5×10^{-4} M.

b. No inhibition observed at inhibitor concentrations up to 1.25×10^{-3} M.

precipitate formed, and there was only a slight change in the inhibitory power of the inhibitor solution (Table 6). Later sections in this thesis will discuss the preparation, characterization, and the inhibition of urease by the oxidized heterocyclic mercaptans.

D. Effect of Reducing Agents on the Inhibition of Urease by the Heterocyclic Mercaptans.

Urease pretreated with either sodium sulfite or 2-mercaptoethanol was unaffected by any of the heterocyclic mercaptans (Table 7). Thus, under reducing conditions none of the heterocyclic mercaptans are effective as urease inhibitors. The hydrogen peroxide treated preparations of rhodanine and 5-amino-1,3,4-thiadiazole-2-thiol were used because often the unoxidized preparations of these compounds were not inhibitory. The thiol-disulfide system is known to be quite sensitive to redox conditions, and mild reducing agents such as sodium sulfite or 2-mercaptoethanol can either break or prevent the formation of a disulfide bond (Boyer, 1960).

E. Dialysis Studies

The activity of a urease preparation that has been inhibited by a heterocyclic mercaptan can be partially restored if the enzyme-inhibitor solution is dialyzed against a reducing agent (Table 8). 2-Mercaptoethanol was more effective than sodium sulfite at eliminating the inhibition of urease by three of the inhibitors. Both reducing agents were equally effective for restoring activity to rhodanine-inhibited urease. In all cases no change in activity occurred during dialysis of the urease-inhibitor solutions against the original buffer (20 mM phosphate-1 mM EDTA, pH 7.0) in the

Table 7

Effect of reducing agents on the inhibition of jack bean urease by heterocyclic mercaptans. Inhibitor solvent redistilled ethanol.

Inhibitor	Preparation number	Reducing Agent	Other conditions	I_{50}, M
rhodanine	1E	sodium sulfite	thiazole/H_2O_2 = 1	none [a,d]
rhodanine	1E	2-mercaptoethanol	thiazole/H_2O_2 = 1	none [a,d]
5-amino-1,3,4-thiadiazole-2-thiol	6B	sodium sulfite	thiadiazole/H_2O_2 = 1	none [b,e]
5-amino-1,3,4-thiadiazole-2-thiol	6B	2-mercaptoethanol	thiadiazole/H_2O_2 = 1	none [b,e]
1,3,4-thiadiazole-2,5-dithiol	8A	sodium sulfite	---	none [c,f]
1,3,4-thiadiazole-2,5-dithiol	8A	2-mercaptoethanol	---	none [c,f]
5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione	2C	---	---	3.1 X 10^{-4}
5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione	2C	sodium sulfite	---	none [f]
5-mercapto-3-phenyl-1-3-4-thiadiazole-2-thione	2C	2-mercaptoethanol	---	none [f]

[a] I_{50} for the control is given for preparation 1E in Table 6 (oxidized with H_2O_2).
[b] I_{50} for the control is given for preparation 6B in Table 6 (oxidized with H_2O_2).
[c] I_{50} for the control is given for preparation 8A in Table 2.
[d] No inhibition observed at inhibitor concentrations up to 5.0 X 10^{-5} M.
[e] No inhibition observed at inhibitor concentrations up to 1.0 X 10^{-4} M.
[f] No inhibition observed at inhibitor concentrations up to 1.25 X 10^{-4} M.

Table 8

Reversibility of urease inhibition by dialysis against reducing agents.

Inhibitor	% of original activity after inhibitors added	Dialysis conditions	% of original activity at various dialysis times				
			1 day	2 days	3 days	5 days	6 days
1,3,4-thiadiazole-2,5-dithiol	0	pH 7.0	0	0	0	0	0
		1 mM sodium sulfite, pH 7.0	5	3	7	11	12
		1 mM mercaptoethanol, pH 7.0	18	27	29	33	36
5-amino-1,3,4-thiadiazole-2-thiol (oxidized, preparation 6B)	0	pH 7.0	0	0	0	0	0
		1 mM sodium sulfite, pH 7.0	10	16	22	24	27
		1 mM mercaptoethanol, pH 7.0	33	56	64	71	78
5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione	0	pH 7.0	0	0	0	0	0
		1 mM sodium sulfite, pH 7.0	19	29	33	39	41
		1 mM mercaptoethanol, pH 7.0	42	62	70	80	86
rhodanine (oxidized, preparation 1E)	20	pH 7.0	18	15	15	19	19
		1 mM sodium sulfite, pH 7.0	40	54	59	66	71
		1 mM mercaptoethanol, pH 7.0	44	56	63	68	75

Four urease solutions [0.2 S.U./ml, dissolved in 20 mM phosphate-1 mM EDTA buffer (pH 7.0)] were each treated with a different inhibitor. Each enzyme-inhibitor solution was assayed for residual activity, divided into three fractions, and each fraction was dialyzed against a different buffer system for five days. At various time intervals the urease activity was determined. After five days all the dialysis buffers were changed to 20 mM phosphate-1 mM EDTA buffer (pH 7.0).

absence of reducing agents (Table 8). Thus, the inhibition of urease by the heterocyclic mercaptans is irreversible under the assay conditions that were employed for the inhibition studies. Total inhibition of urease by rhodanine was not achieved even though an inhibitor concentration (6.0×10^{-5}M) considerably in excess of the I_{50} value was employed. The inhibition of urease by 1,3,4-thiadiazole-2,5-dithiol was less readily reversed than inhibition caused by the other compounds (Table 8). 1,3,4-Thiadiazole-2,5-dithiol has two mercapto groups and could form polymers via disulfide linkages with several urease molecules to produce an insoluble precipitate that is irreversibly inactivated.

The dialysis buffers were changed to 20 mM phosphate-1 mM EDTA buffer (pH 7.0) after 5 days of dialysis in order to determine if removal of the reducing agents would affect the enzymic activity. Sulfite ions inhibit urease, and the removal of the sodium sulfite should cause an increase in activity. However, for both reducing agents, the removal of the reducing agent had no effect on the urease activity (Table 8).

Since urease that has been inhibited by heterocyclic mercaptans can be restored to partial activity by reducing agents, it is likely that inhibition may occur via formation of a disulfide bond between the mercapto group of a heterocyclic mercaptan and a sulfhydryl group of urease. In the next section the following topics will be discussed:

1. how a disulfide bond can be formed;
2. a proposed mechanism of inhibition;
3. evidence for the inhibition mechanism.

F. Inhibition of Urease by Heterocyclic Disulfides

The previous studies concerning the effect of reducing agents on the inhibition of urease by the heterocyclic mercaptans, implicate the formation of an inhibitor-urease disulfide bond. Inhibition may be caused in two ways. A disulfide bond can be formed between a mercapto group of the inhibitor and a sulfhydryl (mercapto) group at the active site of urease, thus blocking the active site. A disulfide bond can also be formed at a location other than the active site, and the inhibition caused by a change in the conformation of the enzyme. However, a thiol will not react with another thiol if no external oxidant is present, because a disulfide bond is in a higher oxidation state than two thiols. It is possible for a thiol to react with a disulfide by thiol-disulfide exchange to produce a new disulfide product as outlined in the following reaction (Boyer, 1960):

$$\text{R-SH} + \text{R}'\text{-S-S-R}' \longrightarrow \text{R-S-S-R}' + \text{R}'\text{-SH}$$

Also, urease has been purified by thiol-disulfide exchange using a Sepharose-glutathione column with 2-mercaptopyridine bound to glutathione by a disulfide linkage (Carlsson *et al*, 1974b).

If a thiol-disulfide exchange reaction is responsible for the inhibition of urease by the heterocyclic mercaptans, then the disulfides of these compounds should be even more effective inhibitors. 5-Amino-1,3,4-thiadiazole-2-thiol and 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione were oxidized to their disulfides which are, 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide and 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide, respectively. The properties of the disulfides and the verification of their structures are given in Section IV of this thesis. 1,3,4-Thiadiazole-2,5-dithiol was not considered because it is disubstituted and upon oxidation could form higher polymers that would be more difficult to study.

The I_{50} values for the inhibition of jack bean urease by 2,2'di(5-amino-1,3,4-thiadiazole) disulfide and 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide are lower than the corresponding mercaptans by factors of 500 and 100 respectively (Table 9). The I_{50} values of a number of different disulfide preparations are also fairly reproducible (Table 9, footnotes d and e). 2,2'-Di(5-amino-1,3,4-thiadiazole) disulfide was dissolved in 95% ethanol containing 0.5% DMSO because the inhibitor was sparingly soluble in ethanol, and DMSO increased the solubility of the compound.

5,5'-Di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide is equivalent to acetohydroxamic acid as a urease inhibitor and 2,2'-di (5-amino-1,3,4-thiadiazole) disulfide is more potent than acetohydroxamic acid (Table 9). Acetohydroxamic acid, a well-known urease inhibitor was included in this study for comparison. Although the I_{50} value for acetohydroxamic acid listed in Table 9 is different from the value reported by Kobashi et al, (1971), the experimental conditions are quite different in both cases.

The extremely potent anti-urease activity of the heterocyclic disulfides and the effect of reducing agents on the inhibition of urease supports the hypothesis of inhibition mediated by a thiol-disulfide exchange. Thus, heterocyclic mercaptans are not inhibitory in either the thione or thiol form, but only when they are in the

Table 9

Comparison of the inhibitory power of acetohydroxamic acid, two of the heterocyclic mercaptans and the disulfides of the two heterocyclic mercaptans.

Inhibitor	Inhibitor preparation number	Inhibitor solvent	Composition of inhibitor preparation	I_{50}
acetohydroxamic acid	1F	water	--	3.0×10^{-6} [a]
5-amino-1,3,4-thiadiazole-2-thiol	6B	ethanol-redistilled	thiadiazole/H_2O_2 = 1	5.2×10^{-5} [b]
5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione	2C	ethanol-redistilled	--	3.1×10^{-4} [c]
2,2'-di(5-amino-1,3,4-thiadiazole) disulfide	--	ethanol-redistilled + 0.5% DMSO	--	1.1×10^{-7} [d]
5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide	--	ethanol-redistilled	--	3.1×10^{-6} [e]

a. Literature value: 6.2×10^{-7} M (Kobashi et al, 1971).

b. Lowest I_{50} value for this compound, taken from Table 6.

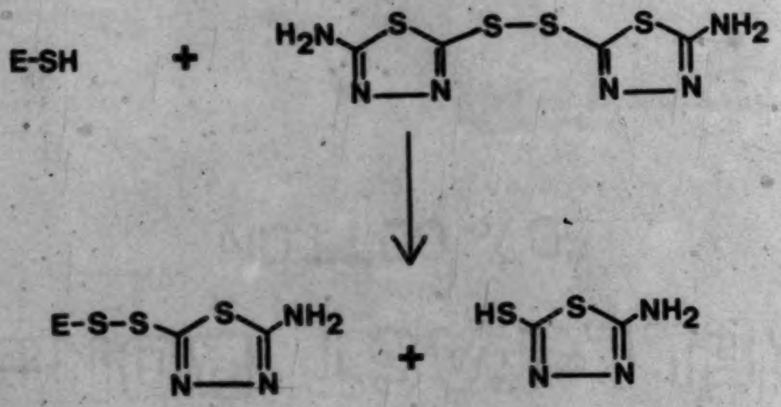
c. Lowest I_{50} value for this compound, taken from Table 7.

d. Average of I_{50} values obtained using five separate inhibitor preparations. Coefficient of variation = 15%.

e. Average of I_{50} values obtained using four separate inhibitor preparations. Coefficient of variation = 25%.

disulfide or polysulfide form. The inhibition of urease by the heterocyclic mercaptans can be attributed to trace quantities of the corresponding disulfides or polysulfides present as impurities. The increased inhibitory power of the oxidized mercaptans (Table 6) was caused by the higher concentrations of the disulfides which had been formed during oxidation. The ability of sodium hydroxide to eliminate the inhibitory power of 1,3,4-thiadiazole-2,5-dithiol can be explained by the assumption that the disulfides or polysulfides had been cleaved by the sodium hydroxide. It has been known for some time that the hydroxide ion can cleave disulfane derivatives (Schmidt, 1965).

The inhibition of urease by 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide can be represented by the following scheme:



The same scheme can be postulated for the disulfides of rhodanine and 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione.

1,3,4-Thiadiazole-2,5-dithiol is disubstituted and may form higher polymers, so there could be more than one inhibitory species.

G. Time Studies

The disulfides of two of the heterocyclic mercaptans, 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide and 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide were employed for the time studies because the disulfide rather than the mercaptan is the active inhibitory form. For similar reasons an oxidized ethanolic solution of rhodanine was used in the time studies.

When the inhibitors were added to solutions of jack bean urease, the inhibition of the urease increased for 2-3 hours and then remained constant (Figures 5, 6, 7 and 8). Thus, the preincubation time of 4 hours that was chosen for the inhibition studies was sufficient to allow the enzyme-inhibitor system to reach equilibrium. The rate at which these compounds inactivate urease was proportional to the initial inhibitor concentration (Figures 5, 6, 7 and 8).

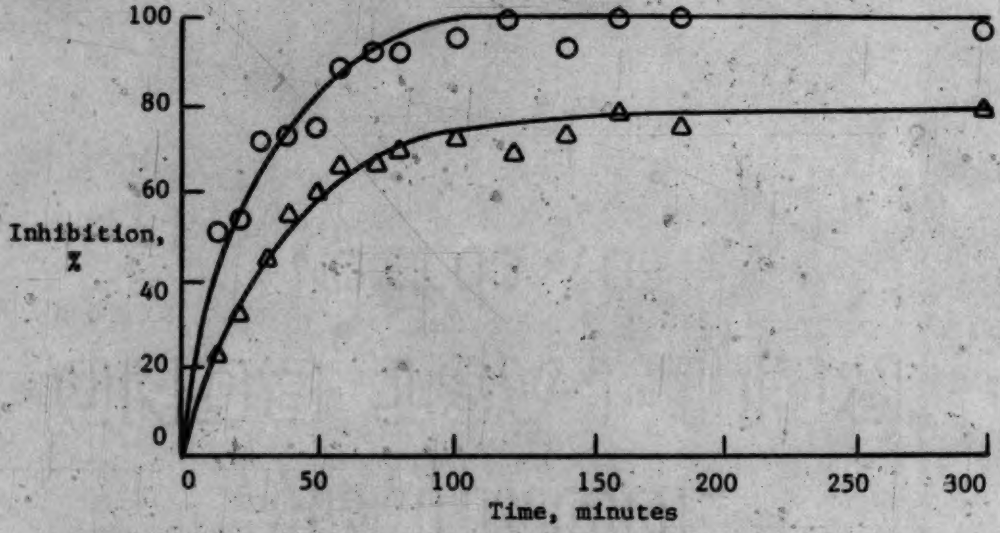


Figure 5: Effect of time on the inhibition of jack bean urease by 1,3,4-thiadiazole-2,5-dithiol. Preparation #10A.

Inhibitor concentration 2.4 x 10^{-5}M —△—△—△—

Inhibitor concentration 4.8 x 10^{-5}M —○—○—○—

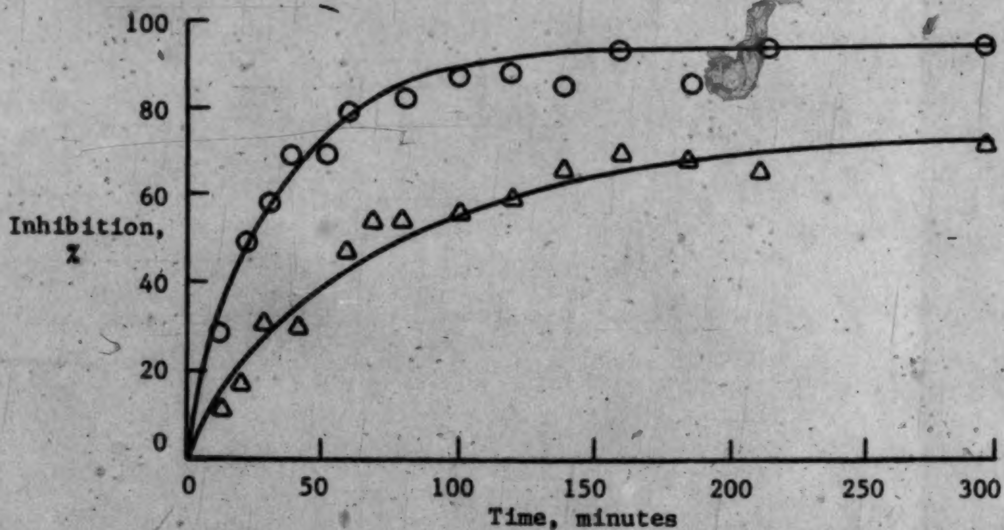


Figure 6: Effect of time on the inhibition of jack bean urease by 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide.

Inhibitor concentration 3.5 x 10^{-6}M —△—△—△—

Inhibitor concentration 7.0 x 10^{-6}M —○—○—○—

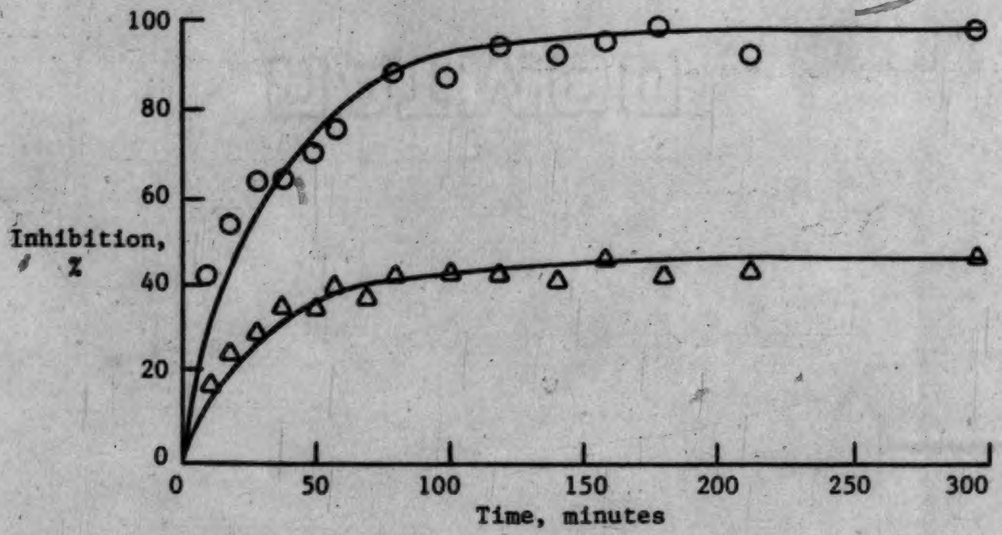


Figure 7: Effect of time on the inhibition of jack bean urease by 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide.

Inhibitor concentration 1.0 x 10^{-7}M —△—△—△—

Inhibitor concentration 2.0 x 10^{-7}M —○—○—○—

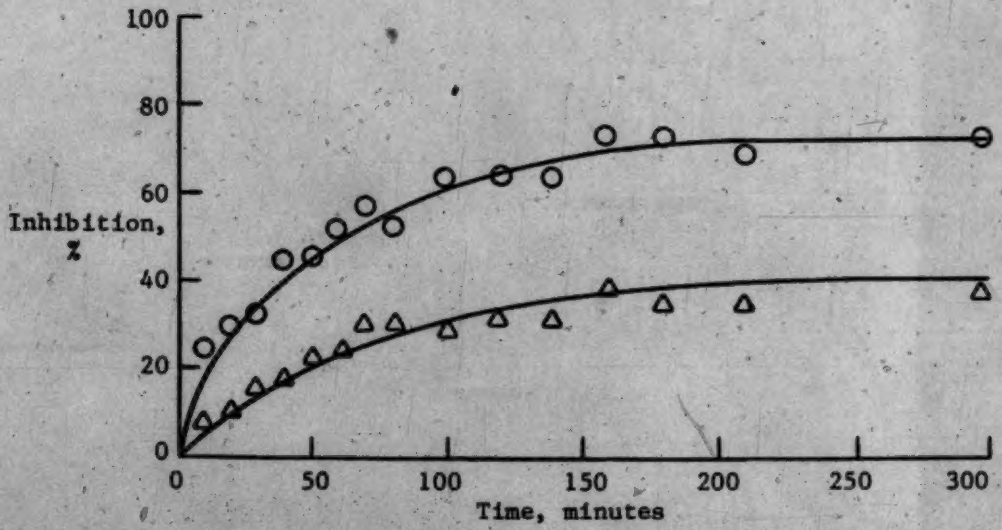


Figure 8: Effect of time on the inhibition of jack bean urease by rhodanine. Preparation #1E. (oxidized with H_2O_2).

Inhibitor concentration 1.5 x 10^{-5}M —△—△—△—

Inhibitor concentration 3.0 x 10^{-5}M —○—○—○—

III. Evaluation of Various Compounds as Soil Urease Inhibitors

The compounds evaluated in this study were a number of heterocyclic mercaptans, two disulfides, and a number of known urease inhibitors such as phenols, benzoquinones, hydroxamates, thiourea, and related compounds.

Only the first two heterocyclic mercaptans listed in Table 10 inhibit soil urease to any extent. In fact, 2-mercaptobenzoxazole increased the rate of urea hydrolysis in soil. The potassium salt of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione was found to be ineffective as a soil urease inhibitor although it was an effective inhibitor of jack bean urease in phosphate buffer at pH 7.0 (Table 3). The most effective of the heterocyclic mercaptans, 1,3,4-thiadiazole-2,5-dithiol was studied in more detail. Inhibition was linear with inhibitor concentration up to 40 ppm of 1,3,4-thiadiazole-2,5-dithiol, but levelled off somewhat at higher inhibitor concentrations (Figure 9). The inhibition of soil urease by 1,3,4-thiadiazole-2,5-dithiol increases with higher moisture content up to a moisture concentration of 40% (w/w of soil) and is then unaffected by moisture content between 40 and 120% (Figure 10).

The experiments with jack bean urease, previously discussed in this thesis, prove that these heterocyclic mercaptans are more effective after they have been oxidized to the corresponding disulfide. However, the disulfides of 5-amino-1,3,4-thiadiazole-2-thiol and 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione are both less effective than expected (Table 10) as soil urease inhibitors. The following reasons could explain the observed discrepancy: (1) the environment

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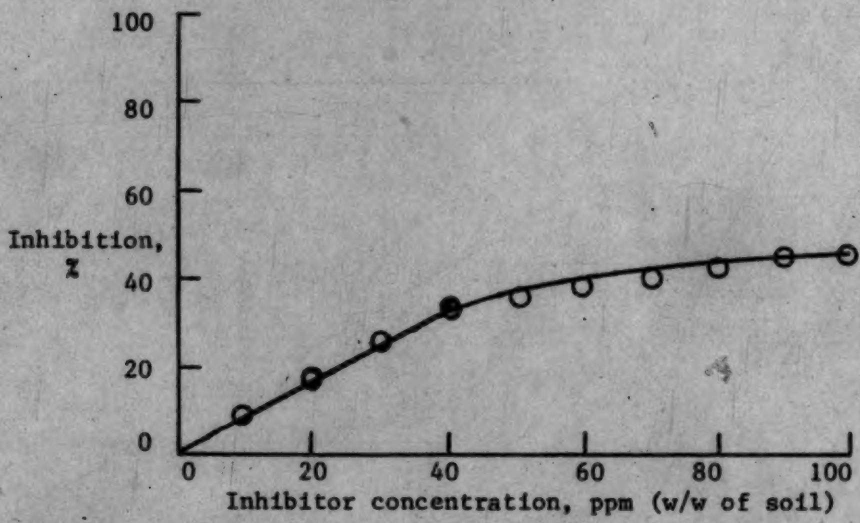


Figure 9: Effect of inhibitor concentration on the inhibition of soil urease activity by 1,3,4-thiadiazole-2,5-dithiol. Substrate concentration 400 ppm urea-N, soil moisture 32%, temperature 25°C, and an incubation time of 24 hr.

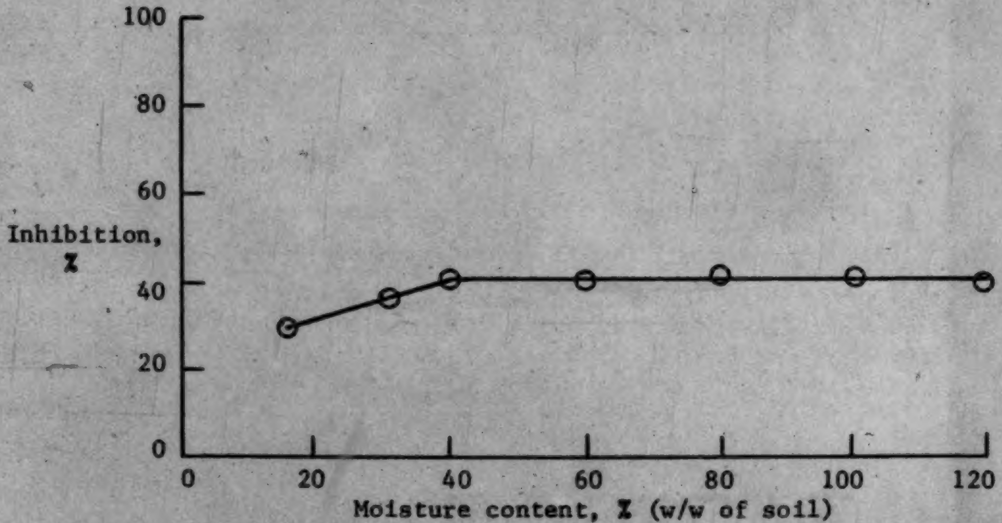


Figure 10: Effect of moisture content on the inhibition of soil urease activity by 1,3,4-thiadiazole-2,5-dithiol. Substrate concentration 400 ppm urea-N, inhibitor concentration 50 ppm, temperature 25°C, and an incubation time of 24 hr.

Table 10

Effect of Various Compounds on Soil Urease Activity

A. Heterocyclic Sulfur Compounds

Compound	% Inhibition
* 1,3,4-thiadiazole-2,5-dithiol	46
5-amino-1,3,4-thiadiazole-2-thiol	27
2-mercapto-1-methyl imidazole	13
* rhodanine	10
* neothiocyanic acid	9
* trithiocyanuric acid	8
* 2-mercapto-4(3H)quinazolinone	0
2-mercaptopyridine-N-oxide, sodium salt	0
5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione, potassium salt	0
* 2-mercaptobenzimidazole	0
* 2-mercaptobenzathiazole	0
* 2-mercaptobenzoxazole	-16
* 2,6-dimercaptopurine	0
2-thiazoline-2-thiol	0
* 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide	11
* 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide	6

B. Phenols and Benzoquinones

Compound	E°' (pH = 7.0)‡	% Inhibition
hydroquinone	(0.284)	88
catechol	(0.378)	47
4-chlorophenol		14
phenol		10
* p p' bephenol		5
* 4-phenylphenol		5
pyrogallol		2
resorcinol		0
1-napthol		1
1,4-benzoquinone	(0.284)	88
2,5-dimethyl-1,4-benzoquinone	(0.170)	50
2,6-dimethyl-1,4-benzoquinone		45
* 2,6-dichloroquinone-4-chloroimide		30
* tetrachloro-1,2-benzoquinone	(0.290)	6

Table 10 (continued)

C. Urea Analogs, Thiourea Derivatives and Hydroxamates

Compound ≠	% Inhibition
acetohydroxamic acid	17
hydroxyurea	16
N'-methyl-N-hydroxyurea	15
thiourea	9
* CDU	5
acetaldehyde thiourea	0
sodium oxamate	0

D. Aldoximes and Imides

Compound	% Inhibition
formamidine acetate	0
acetamidine acetate	0
acetamidoxime	3
N-hydroxysuccinimide	0
* phthalimide	0

E. Other Heterocyclic Nitrogen Compounds

Compound	% Inhibition
4-amino-1,2,4-triazole	4
5-aminotetrazole monohydrate	4
* 2-aminothiazole	4
2-amino-5-methyl-1,3,4-thiadiazole	4
3-pyridinol	2
2(1H)-pyridone	0
4(1H)-pyridone	0
cyanuric acid	0
2-sulfonamide-5-acetamido-1,3,4-thiadiazole	0

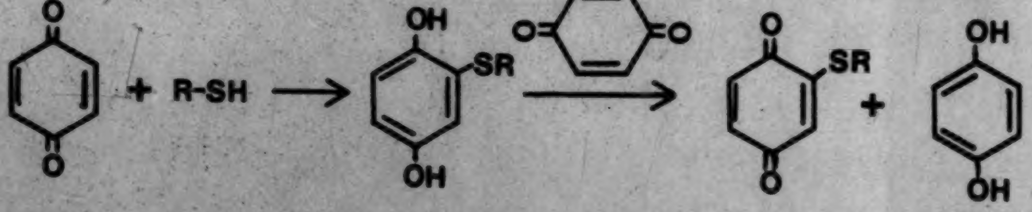
The experimental conditions are described in the materials and methods section. In the absence of inhibitors 312 ppm of the added urea-N was hydrolyzed.

* Due to the low solubility of these compounds in water they were applied as a slurry.

≠ $E^{o\prime}$ values obtained from Webb, 1966.

of soil urease is different than that of urease in solution and the mercapto compounds could inhibit soil urease by a different mechanism, i.e. a metal-thiazole complex or chelation of the nickel atoms in the urease molecule. (2) The higher solubility of the thiol compounds may allow them to diffuse more readily through the soil to the active site of urease where other soil constituents could act as oxidants and catalysts to promote the formation of a disulfide bond between the thiol group and the essential sulfhydryl group of urease.

Dihydric phenols and quinones have been shown to be effective inhibitors of soya bean urease (Quastel, 1933), jack bean urease (Grant and Kinsey, 1933), and soil urease (Bremner and Douglas, 1971a, Bundy and Bremner, 1973). The dihydric phenols are only effective inhibitors after they have autooxidized to the quinone form (Quastel, 1933). The two most probable mechanisms for the inhibition of urease by quinones are: (1) oxidation of essential sulfhydryl groups and (2) reaction of the quinones with sulfhydryl groups by 1,4-addition (Hoffmann-Ostenhof, 1963, Webb, 1966). Thiols react readily with 1,4-benzoquinone by 1,4-addition to give a monosubstituted hydroquinone which is subsequently oxidized by excess 1,4-benzoquinone to a substituted benzoquinone (Burton and David, 1953, Schubert, 1947, Snell and Weissberger, 1939) as illustrated by the following scheme:



In the presence of oxygen, hydroquinone will be oxidized again to 1,4-benzoquinone and the addition cycle can repeat itself.

At increasing pH values the redox potential decreases, but the maximum inhibition of urease by quinones occurs between pH 7.0 and 8.0. If oxidation of sulfhydryl groups were responsible for the inhibition of urease by quinones the inhibition should increase with decreasing pH. The addition mechanism for the inhibition of urease by quinones is the most likely one (Webb, 1966). In the case of either oxidation or the formation of an addition product, inhibition can still be correlated with the redox potential of the quinone because a redox step is also involved in the formation of an addition product (Webb, 1966).

The redox potentials of several of the quinones and the quinone forms corresponding to the dihydric phenols in Table 10 are listed. Inhibition of soil urease by both hydroquinone and 1,4-benzoquinone are identical as expected since hydroquinone oxidizes very readily to the quinone form. Tetrachloro-1,2-benzoquinone has a fairly high redox potential but is ineffective as a soil urease inhibitor. Since all four of the available positions on tetrachloro-1,2-benzoquinone are occupied by chlorine atoms, it would not be able to form an addition product with a thiol. Phenols which are unable to be oxidized to the quinone form will also be ineffective as urease inhibitors.

Acetohydroxamic acid and hydroxyurea both inhibit soil urease but are much less effective than some of the other compounds studied (Table 10).

The aldoxime class of compounds were considered because of their structural similarity to the hydroxamates. However, the aldoximes and the imide class of compounds, as well as various other heterocyclic nitrogen compounds were found to be ineffective as soil urease inhibitors.

The most promising compounds as soil urease inhibitors are several of the phenols and benzoquinones (Table 10) and the first two heterocyclic mercaptans listed in Table 10. Phenols and benzoquinones are strong irritants (Webb, 1966) and may be of limited practical use as urease inhibitors. Although 1,3,4-thiadiazole-2,5-dithiol is somewhat less effective as a soil urease inhibitor than some of the benzoquinones, it is much less toxic and thus is the most suitable inhibitor for practical applications.

A considerable volume of work has been performed on the inhibition of nitrification in soil in order to improve the efficiency of nitrogen fertilizers (Fujikawa, 1971). 1,3,4-Thiadiazole-2,5-dithiol has been shown to inhibit nitrification in pure culture (Fujikawa, 1971) and 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide has also been shown to completely inhibit nitrification in pure culture[a] at concentrations as low as 0.1 ppm (w/v of culture solution). It is possible that the inhibition of nitrification by the heterocyclic mercaptans could also occur by a thiol-disulfide exchange in a manner analogous to the inhibition of urease by these compounds. The heterocyclic mercaptans and their disulfides are worthy of further investigation with regard to both the inhibition of urease activity and the inhibition of nitrification.

[a] *unpublished work in our laboratory.*

IV. Synthetic Chemistry

A. Preparation of 3,4-Dimethyl-2,5-Dithione-1,3,4-Thiadiazolidene

The synthesis was carried out as indicated in the Materials and Methods section using 0.1 moles of the starting materials. The crude product was recrystallized from ethanol to yield white needles of 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene.

Theoretical yield = 178 X 0.1 = 17.8 g

Actual yield = 4.7 g

$$\% \text{ Yield} = \frac{4.7}{17.8} \times 100 = 27\%$$

(Lit. value 50%, Thorn, 1960)

The yield was somewhat lower than the literature value because some product was lost during purification. The melting point was identical to the literature value, and the molar extinction coefficients of the ultraviolet absorption peaks for 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene were 17 - 34% higher than the literature values (Figure 11). The singlet peak at 3.92 ppm in the n.m.r. spectrum corresponds to the methyl protons of the compound (Figure 11). The parent ion (m/e 178) in the mass spectrum corresponds to the molecular weight of the compound (Table 11).

Thus, the properties listed in Figure 11 and Table 11 are consistent with the proposed structure of 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene.

B. Properties of Two of the Heterocyclic Mercaptans and the Corresponding Disulfides.

5-Amino-1,3,4-thiadiazole-2-thiol and 5-mercapto-3-phenyl-2-thione-1,3,4-thiadiazole were each oxidized to their respective disulfides as described in the Materials and Methods section. 1,3,4-

M.P. = 168°C (Lit. value 168-169°C, Thorn, 1960)

n.m.r. Spectrum

δ (60 MHz, in $CDCl_3$) , 3.92

(S, 6H, H_3C-N-N-CH_3) internal standard, TMS

TMS = tetramethylsilane

s = singlet

Ultraviolet Spectrum (in 95% ethanol)

λ_{max} 269 (268) nm 328 (327) nm

ϵ 14,300 (10,700) 14,000 (12,000)

ϵ = molar extinction coefficient

Literature values in brackets (Thorn, 1960)

Infrared Spectrum (in $CHCl_3$)

Absorption bands, cm^{-1}	Assignment (Colthup, Daly and Wiberley, 1975, Dyer, 1965)
930 M	C-H rocking
1100 S	C=S stretching
1200-1280 M	C-N
1380 M	C-H bending
1480 S	C-H bending
3000 W	C-H stretching

S = strong M = medium W = weak

Structure

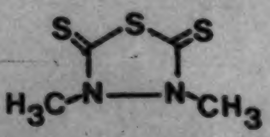


Figure 11: Properties of 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene.

Table 11

Mass Spectrum of 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene

m/e	% of Base Peak
15	7.5
28	5.7
42	9.7
43	39
44	6.3
45	17
46	6.5
58	13
61	6.2
64	45
72	35
73	32
74	11
76	11
101	9.9
105	9.4
178	100 (Base and Parent)
179	7.5 (P+1)
180	14 (P+2)
181	0.92 (P+3)

Thiadiazole-2,5-dithiol was not considered because it is disubstituted with mercapto groups, and upon oxidation could form higher polymers that would be more difficult to study.

The infrared spectra of the monomers and dimers of these two compounds are shown in Figures 12, 13, 14 and 15, and the exact absorption frequencies are listed in Appendices III, IV, V and VI. The ultraviolet spectra are shown in Figures 16, 17 and 18. No ultraviolet spectrum could be obtained for 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide because it is only sparingly soluble in ethanol, the solvent of choice for these studies. An ultraviolet spectrum of 2,5-dithione-3,4-dimethyl-1,3,4-thiadiazolidene is shown for comparison (Figure 16). The n.m.r. spectra are presented in Figures 19, 20, 21 and 22, and Figure 23 is an n.m.r. spectrum of the solvent ($DMSO_{d6}$) alone. The exact values of the n.m.r. absorptions, the molar extinction coefficients of the ultraviolet maxima and additional properties of these compounds are listed in Figures 24, 25, 26 and 27. The mass spectra are listed in Tables 12, 13, 14, 15, 16 and 17. Tables 16 and 17 each contain a mass spectrum of one of the disulfides that was purified by sublimation.

The spectroscopic results confirmed that the monomers have been converted to the corresponding dimers and also provides evidence for the thione-thiol tautomerism of these compounds. The infrared spectra of 5-amino-1,3,4-thiadiazole-2-thiol and its dimer in the solid phase (KBr pellets) differ somewhat from each other as expected (Figures 12 and 13). The two features of the infrared spectrum which suggest that 5-amino-1,3,4-thiadiazole-2-thiol is in

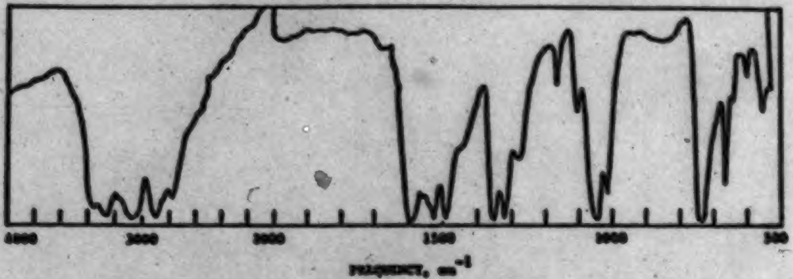


Figure 12: Infrared spectrum of 5-amino-1,3,4-thiadiazole-2-thiol in a KBr pellet.

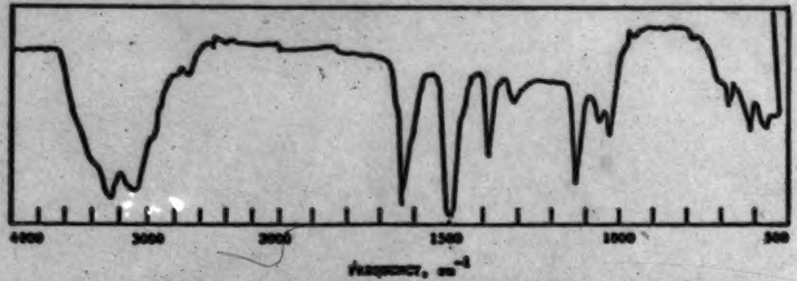


Figure 13: Infrared spectrum of 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide in a KBr pellet.

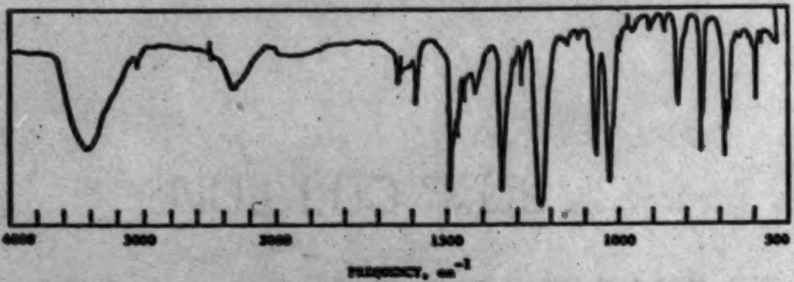


Figure 14: Infrared spectrum of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione in a KBr pellet.

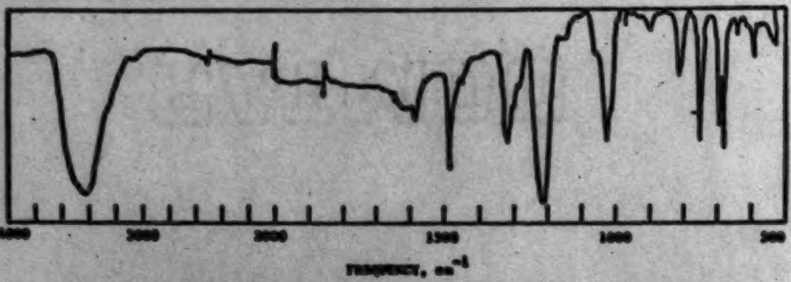


Figure 15: Infrared spectrum of 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide in a KBr pellet.

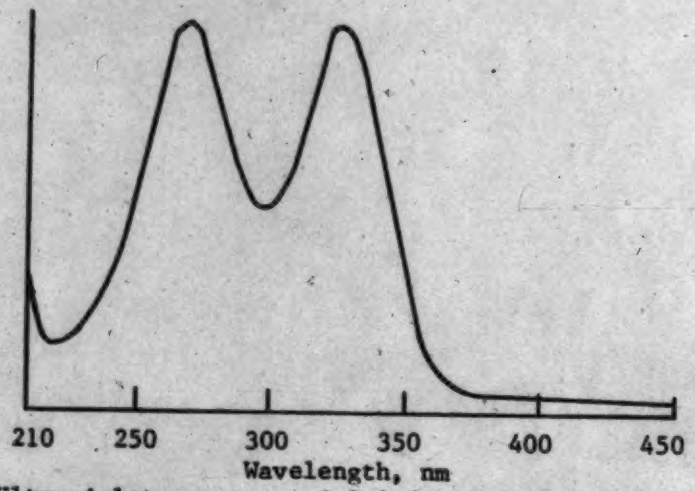


Figure 16: Ultraviolet spectrum of 3,4-dimethyl-2,5-dithione-1,3,4-thiadiazolidene in 95% ethanol.

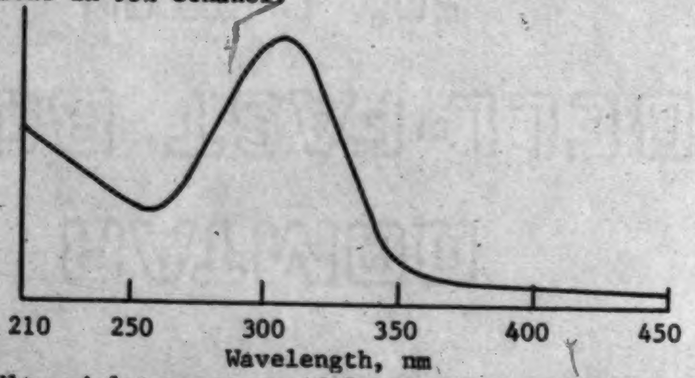


Figure 17: Ultraviolet spectrum of 5-amino-1,3,4-thiadiazole-2-thiol in 95% ethanol.

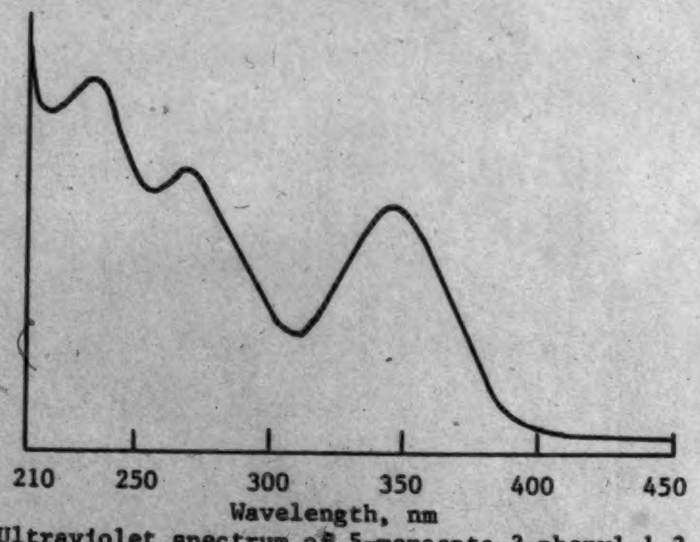


Figure 18: Ultraviolet spectrum of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione in 95% ethanol.

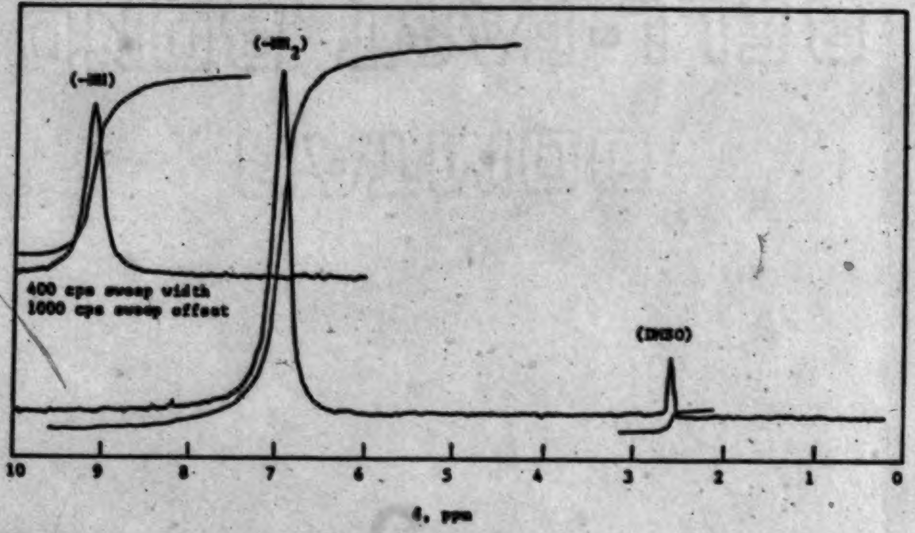


Figure 19: n.m.r. spectrum of 5-amino-1,3,4-thiadiazole-2-thiol in DMSOd$_6$.

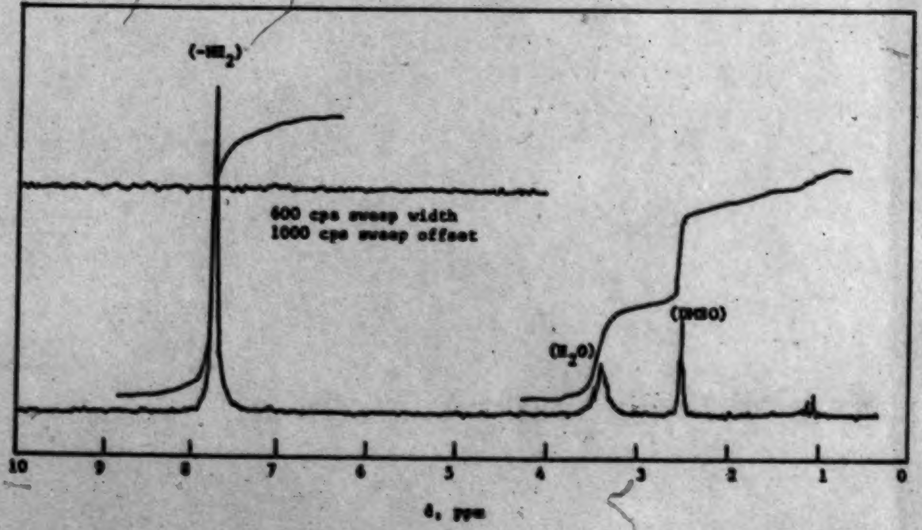


Figure 20: n.m.r. spectrum of 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide in DMSOd$_6$.

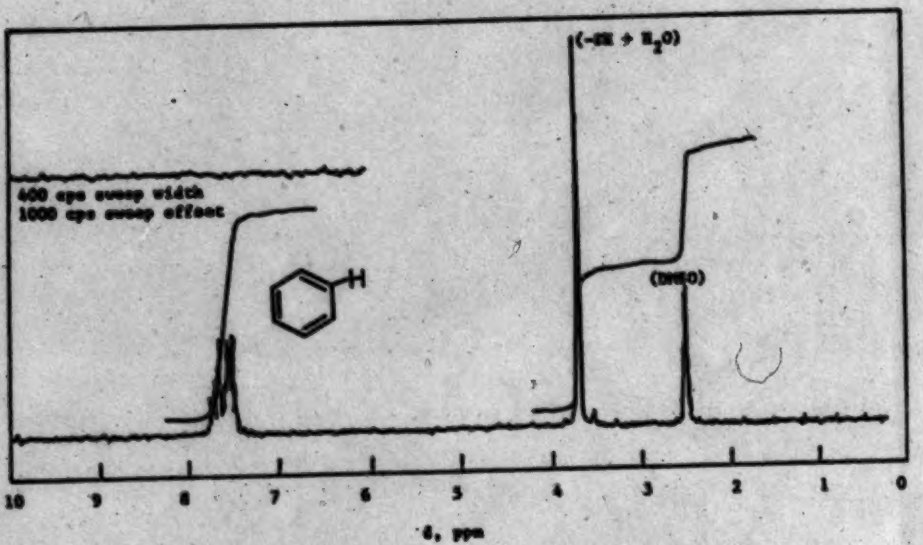


Figure 21: n.m.r. spectrum of 5-mercapto-3-phenyl-1,3,4-thiadiazole -2-thione in DMSOd$_6$.

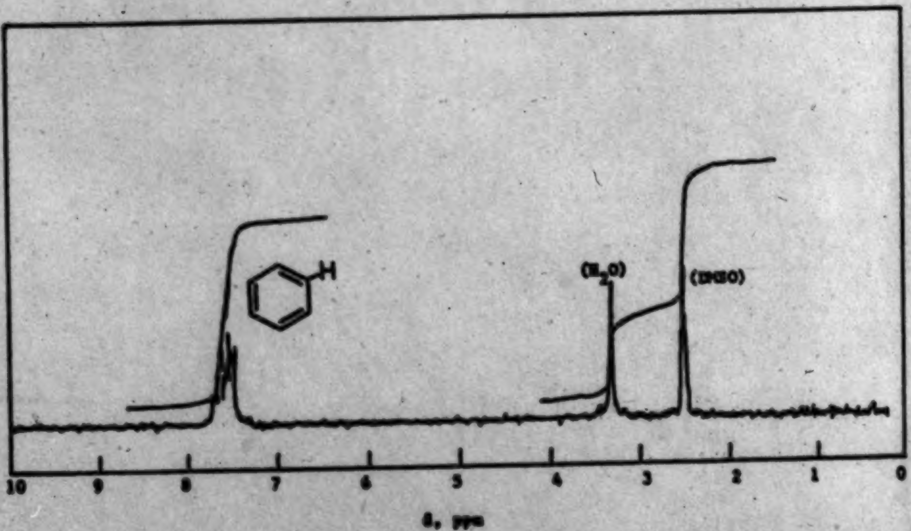


Figure 22: n.m.r. spectrum of 5,5'-di(3-phenyl-1,3,4-thiadiazole -2-thione) disulfide in DMSOd$_6$.

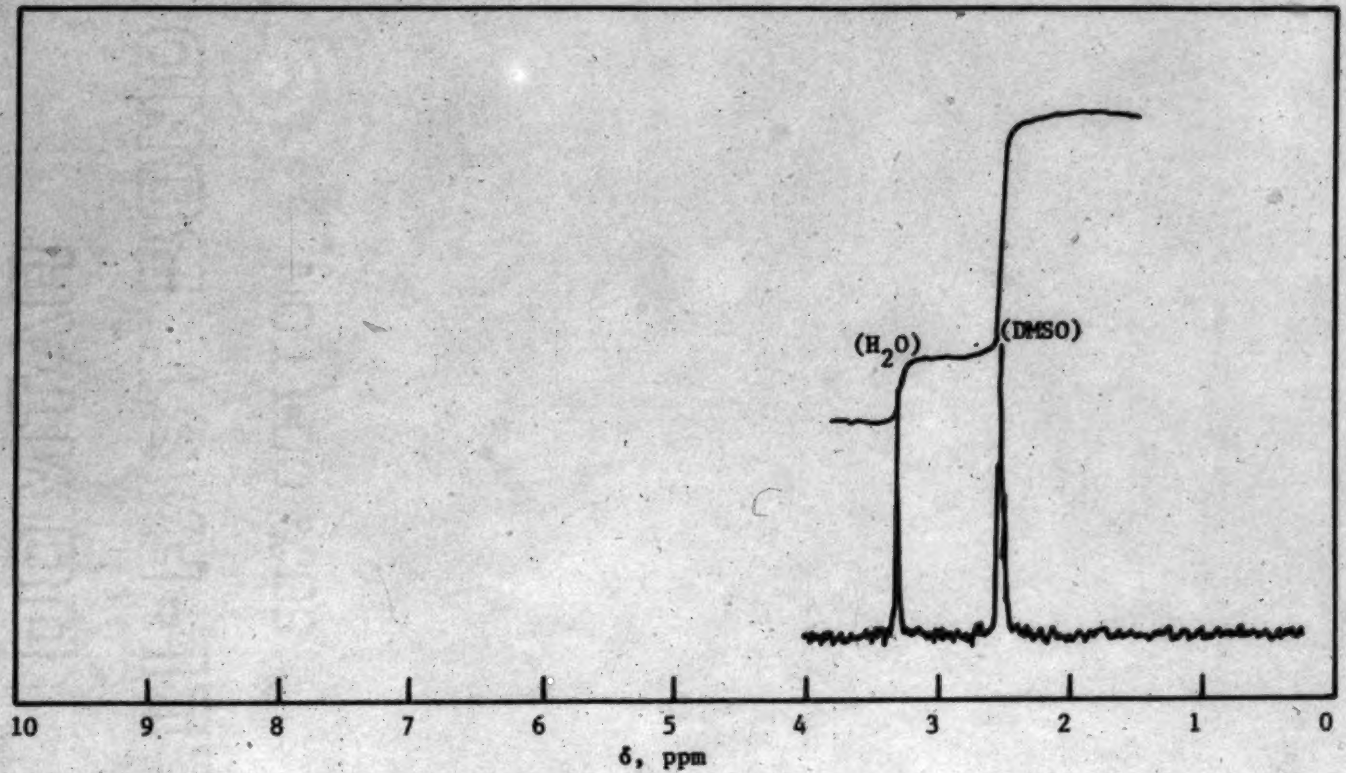


Figure 23: n.m.r. spectrum of the solvent (DMSOd$_6$).

M.P. = 236-238°C (decomposes)

Fine white crystals.

n.m.r. Spectrum:

(100 MHz, in $DMSO_{d6}$)

δ 6.96 (S, 2H, $-\underline{NH_2}$), 13.13 (S, 1H, $-\overset{\prime\prime}{N}-\overset{\prime}{N}-\underline{H}^{*}$)

internal standard, TMS

S = singlet

* The compound was assumed to be in the thione tautomer in DMSO. The chemical shift is consistent with a proton bound to a heterocyclic nitrogen atom (p 209, Jackman and Sternhell, 1969).

Ultraviolet Spectrum:

λ_{max} = 310 nm

ε = 9,300

ε = molar extinction coefficient

Structure

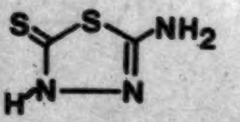


Figure 24: Properties of 5-amino-1,3,4-thiadiazole-2-thiol.

M. P. = 236-238°C (decomposes)

Fine yellow crystals.

n.m.r. Spectrum:

(100 MHz, in $DMSO_{d6}$)

δ 7.72 (S, 4H, $N\underline{H_2}$) internal standard, TMS

S = singlet

Structure

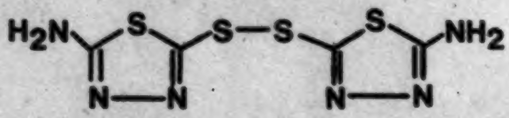


Figure 25: Properties of 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide.

M.P. = 85-87°C

Fine white crystals.

n.m.r. Spectrum:

(100 MHz, in $DMSO_{d6}$)

δ 7.60 (m, 5H, ϕ-$\underline{H}$), 3.72 (S, 1H, -SH)*

internal standard, TMS

s = singlet
m = multiplet

* To obtain the integration for this peak, the value for the water peak in Figure 22 was subtracted from the combined ($SH + H_2O$) peak in Figure 21.

Ultraviolet Spectra:

(a) Spectrum of the potassium salt:

λ_{max} = 236 nm 273 nm 347 nm

ϵ = 16,200 ± 830 11,800 ± 320 9,700 ± 50

(b) Spectrum in the presence of a reduing agent: (molar ratio of 2-mercaptoethanol/thiazole = 10).

λ_{max} = 239 nm 270 nm 347 nm

ϵ = 14,800 ± 600 11,700 ± 370 9,400 ± 130

ϵ = molar extinction coefficient

Structure

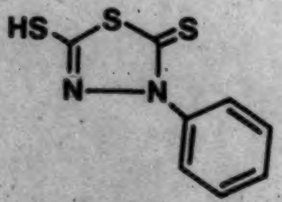


Figure 26: Properties of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione.

M.P. = 133.5-134°C

Orange crystals

n.m.r. Spectrum:

(100 MHz, in $DMSO_{d6}$)

δ 7.62 (m, 10H, ϕ-$\underline{H}$)

internal standard, TMS

m = multiplet

Ultraviolet Spectrum:

λ_{max} =	225 nm	271 nm	344 nm
ϵ =	26,300	19,000	13,800

ϵ = molar extinction coefficient

Structure

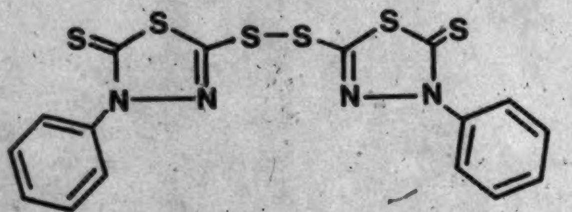


Figure 27: Properties of 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide.

the thione tautomer are: (a) the absence of a thiol peak, (2) the broad =N-H stretching peak at 2270 cm^{-1} (Figure 10). When =N-H stretching occurs between 2800 and 2600 cm^{-1}, there is some hydrogen bonding involved. The disappearance of the peak at 2770 cm^{-1} when the dimer is formed (Figure 13) is also expected if the thione tautomer predominates. This is in agreement with the X-ray crystallography studies of Downie _et al_, (1972) who were able to show that in the solid state 5-amino-1,3,4-thiadiazole-2-thiol is present as the thione tautomer and hydrogen bonding occurs between the proton on the ring nitrogen and a thione group of another molecule. The infrared spectrum of the dimer is consistent with its expected structure (Figure 13). The infrared spectrum of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione has a peak at 2330 cm^{-1} (Figure 14) corresponding to a thiol group. This peak is absent from the infrared spectrum of the dimer (Figure 15) which is additional evidence for the proposed structure. Thus, in the solid state 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione exists as the thiol tautomer.

The ultraviolet spectra did not provide much information with respect to thione-thiol tautomerism. Although the extinction coefficients for 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione and 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide are different, the absorption maxima and the appearance of the spectra are both similar (Figures 18, 26 and 27). The ultraviolet spectra of the potassium salt of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione and the corresponding acid under reducing conditions are identical (Figure 26). It was necessary to add a reducing agent to the acid in order to prevent oxidation and formation of the dimer. The conclusions of Thorn (1960) regarding the thione-thiol tautomerism of 1,3,4-thiadiazole-2,5-dithiol in 95% ethanol were criticized by Sandstrom (1968) as he suggested that the compound exists as a monoanion rather than as a tautomer of the acid.

N.m.r. studies are most commonly carried out in carbon tetrachloride or deuterated chloroform ($CDCl_3$). In this study it was necessary to use deuterated dimethyl sulfoxide ($DMSO_{d6}$) as a solvent because the compounds were insoluble in the other solvents. 5-Amino-1,3,4-thiadiazole-2-thiol was assumed to be present as the thione tautomer when dissolved in DMSO, since the proton (=N-H) which is able to tautomerize absorbs at 13.13 ppm in the n.m.r. spectrum (Figures 19 and 24). Thiol protons usually absorb in the region of 0.9-4.0 ppm and a proton bound to a heterocyclic nitrogen atom can absorb as far downfield as 13.7 ppm (Jackman and Sternhell, 1969). The absence of the additional proton in the n.m.r. spectrum of 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide is supporting evidence for the proposed structure of the dimer (Figures 20 and 25). The n.m.r. spectrum of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione has a multiplet peak at 7.60 ppm corresponding to the phenyl protons, and a singlet peak at 3.72 ppm representing a combined peak of either a mercapto or imino group plus trace amounts of water (Figures 21 and 26). The additional proton carries out an exchange with the protons of the trace amount of water, and thus it was not possible to determine if the proton was on the sulfur or the nitrogen. The n.m.r. spectrum of 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide has a multiplet at 7.62 ppm corresponding to the phenyl protons and additional peaks occur at 2.50 ppm and 3.32 ppm which can be attributed to DMSO and trace amounts of water respectively (Figures 22 and 27). The additional peaks were indentical to the DSMO blank which absorbed at 2.50 ppm and 3.30 ppm respectively (Figure 23).

The mass spectra of 5-amino-1,3,4-thiadiazole-2-thiol and 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione were consistent with the known structures of these compounds (Tables 12 and 14). The mass spectrum of 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide (Table 15) has the correct mass ion (molecular weight 450), but the mass spectrum of 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide is not consistent with the postulated structure (Table 13). The apparent mass ion of 256 for 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide is 8 less than the expected molecular weight. The mass spectra (Tables 16 and 17) of both disulfides that had been purified by sublimation were similar to the original spectrum of 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide (Table 13). Since a mass ion of 256 was found for the sublimated disulfides (Tables 16 and 17), which had molecular weights of 264 and 450 respectively, it is likely that a thermal rearrangement is taking place during sublimation. 2,2'-Di (5-amino-1,3,4-thiadiazole) disulfide could also have rearranged in the mass spectrometer to produce an erroneous spectrum. All of the mass spectra that had an apparent mass ion of 256 also contained significant peaks of m/e values 32, 64, 96, 160 and 192 in common (Tables 13, 16 and 17). The mass ion of 256 and a fragmentation pattern of m/e values that are even multiples of 32 could be

Table 12

Mass spectrum of 5-amino-1,3,4-thiadiazole-2-thiol.

m/e	% of Base Peak	m/e	% of Base Peak
28	37	64	8.1
32	9.1	74	21
43	9.8	76	5.4
45	5.7	133	100 (Base and Parent)
57	37	134	5.1 (P+1)
59	7.8	135	8.7 (P+2)
60	10	136	0.43 (P+3)

Table 13

Mass spectrum of 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide.

m/e	% of Base Peak	m/e	% of Base Peak
27	4.1	74	41
28	42	76	15
29	4.6	77	6.5
32	20	96	7.6
42	15	101	5.5
43	18	128	10
44	7.7	133	100 (Base)
45	11	134	5.8
46	4.2	160	13
47	6.7	192	11
57	67	224	5.0
58	5.0	256	11 (Parent)
59	27	257	0.61 (P+1)
60	24	258	3.8 (P+2)
64	53	259	0.24 (P+3)

Table 14

Mass spectrum of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione.

m/e	% of Base Peak	m/e	% of Base Peak
28	6.8	91	100 (Base)
38	4.7	92	8.9
39	8.6	103	5.8
50	9.6	135	9.2
51	30	150	18
52	4.9	225	6.7
63	7.7	226	49 (Parent)
64	18	227	7.0 (P+1)
65	5.5	228	6.8 (P+2)
76	5.9	229	0.76 (P+3)
77	47		

Table 15

Mass spectrum of 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide.

m/e	% of Base Peak	m/e	% of Base Peak
27	5.3	78	7.1
28	18	91	21
32	6.7	105	7.6
38	4.9	135	97
39	7.9	136	8.8
44	4.1	137	4.7
50	15	149	6.2
51	41	226	6.2
63	5.3	450	7.6 (Parent)
64	7.4	451	1.8 (P+1)
76	5.3	452	2.3 (P+2
77	100 (Base)	453	0.58 (P+3)

Table 16

Mass spectrum of 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide: purified by sublimation.

m/e	% of Base Peak
13	4.3
27	8.6
28	29
29	5.4
32	46
33	4.3
42	13
43	8.9
44	6.8
45	22
46	7.9
47	7.1
58	5.0
59	22
60	13
64	100 (Base)
66	8.9
74	36
96	18
101	50
128	33
130	5.7
160	28
162	6.1
192	13
256	36 (Parent)
257	2.1 (P+1)
258	12 (P+2)
259	0.75 (P+3)

Table 17

Mass spectrum of 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide: purified by sublimation.

m/e	% of Base Peak
32	20
64	100 (Base)
66	12
91	5.6
96	32
98	4.2
128	44
130	7.3
160	21
162	4.9
192	11
256	32 (Parent)
257	2.7 (P+1)
258	11 (P+2)
259	0.61 (P+3)

explained by assuming that the original disulfide decomposed to sulfur (S_8 ring, Molecular weight, 256) and a number of other products.

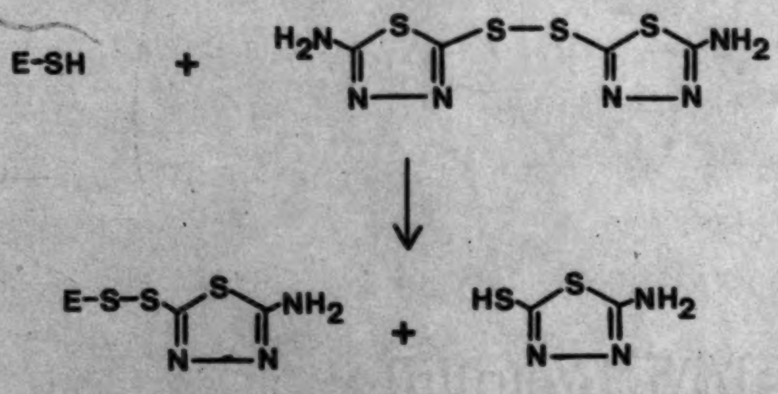
No further attempt was made to characterize the disulfides by mass spectrometry as the infrared and n.m.r. spectroscopy data were deemed sufficient.

CONCLUSIONS

Several important conclusions have been formed regarding the inhibition of both jack bean urease and soil urease by heterocyclic mercaptans. Additional information with respect to the chemical properties of two of these heterocyclic mercaptans has also been obtained.

1. The following heterocyclic mercaptans were found to be effective inhibitors of jack bean urease: 1,3,4-thiadiazole-2,5-dithiol; 5-amino-1,3,4-thiadiazole-2-thiol; 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione; and rhodanine. Inhibition was likely due to trace amounts of the polysulfides or disulfides of these compounds in the mercaptan preparations.

2. The results are consistent with inhibition occurring via a thiol-disulfide exchange reaction between a dimer or polymer of one of these compounds and a sulfhydryl group of urease. The following reaction scheme outlines this hypothesis for the dimer of 5-amino-1,3,4-thiadiazole-2-thiol:



3. Inhibition of jack bean urease by these heterocyclic mercaptans could be prevented by preincubating the enzyme with either 2-mercapto-

ethanol or sodium sulfite. Inhibition could also be partially reversed by dialyzing the inhibited enzyme against either of the same two reducing agents. Two of these heterocyclic mercaptans, 5-amino-1,3,4-thiadiazole-2-thiol and rhodanine, were more potent inhibitors after they had been pretreated with hydrogen peroxide. The effect of reducing and oxidizing agents on inhibition supports the hypothesis of an inhibitor-enzyme disulfide bond.

4. Disulfides of two of the heterocyclic mercaptans were found to be very potent inhibitors of jack bean urease. Unlike the heterocyclic mercaptans, inhibition by the disulfides was found to be reproducible. The inhibition of jack bean urease by the heterocyclic mercaptans is not due to these compounds themselves, but to varying amounts of the corresponding disulfides in the inhibitor preparations. For 1,3,4-thiadiazole-2,5-dithiol there is a possibility of higher polymers being involved. Comparison of the infrared and n.m.r. spectra of these two compounds prove which of the tautomeric forms predominate under several different conditions. 5-Amino-1,3,4-thiadiazole-2-thiol is in the thione tautomer in the solid state, and is probably also in the thione form when dissolved in dimethyl sulfoxide. 5-Mercapto-3-phenyl-1,3,4-thiadiazole-2-thione is in the thiol form in the solid state. Mass spectroscopic studies gave anomalous results which would require further work.

5. The most effective soil urease inhibitors listed in order of effectiveness are: hydroquinone ~ 1,4-benzoquinone > 2,5-dimethyl-1,4-benzoquinone ~ catechol ~ 1,3,4-thiadiazole-2,5-dithiol ~ 2,6-dimethyl-1,4-benzoquinone > 2,6-dichloroquinone-4-chloroimide ~

5-amino-1,3,4-thiadiazole-2-thiol > acetohydroxamic acid. Most of the heterocyclic nitrogen compounds were found to be ineffective. The disulfides of two of the heterocyclic mercaptans were not very effective inhibitors of soil urease, although they were powerful inhibitors of jack bean urease. However, 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide may have some potential as a nitrification inhibitor. Since the phenols and benzoquinones are toxic and probably unsuitable for practical use, 1,3,4-thiadiazole-2,5-dithiol is the most promising soil urease inhibitor.

6. The heterocyclic mercaptans employed in this study would not be effective in environments with low redox potentials because the disulfide bond between the inhibitor and urease is not stable under reducing conditions. Thus, these compounds could not be used as additives to feed urea in order to retard rumen urease activity, because the rumen is anaerobic and reducing.

7. A number of other enzymes are also sulfhydryl dependent such as lactate dehydrogenase, malic dehydrogenase, papain, the aldolases, and the alcohol dehydrogenases (Boyer, 1960). These enzymes were not investigated in this study but would also react with a disulfide inhibitor and might be of interest for future work.

8. Field studies should be carried out to assess the feasibility of using 1,3,4-thiadiazole-2,5-dithiol in a slow-release urea fertilizer. A study should also be initiated to delineate the mechanism by which the heterocyclic disulfides inhibit nitrification, and to determine the potential of these compounds as nitrification inhibitors in field applications.

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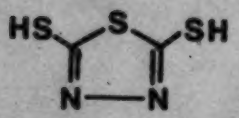
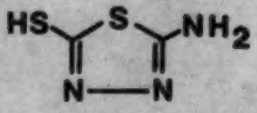
APPENDIX I

COMPOUNDS EVALUATED FOR EFFECTIVENESS AS SOIL UREASE INHIBITORS

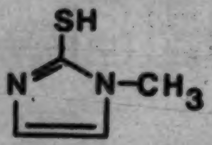
A. Heterocyclic Sulfur Compounds



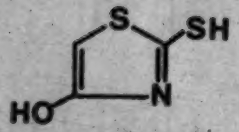
1,3,4-thiadiazole-2,5-dithiol



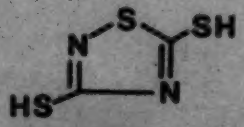
5-amino-1,3,4-thiadiazole
-2-thiol



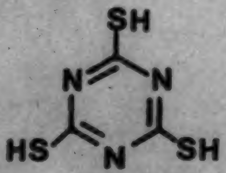
2-mercapto-1-methylimidazole



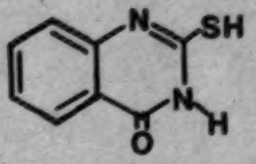
rhodanine



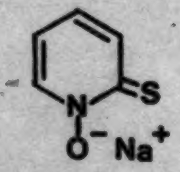
neothiocyanic acid



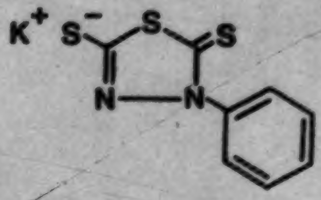
trithiocyanuric acid



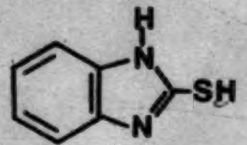
2-mercapto-4(3H)-quinazolinone



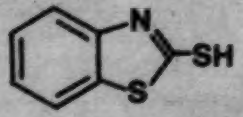
2-mercaptopyridine-N-oxide, sodium salt



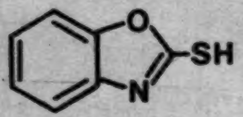
5-mercapto-3-phenyl-1,3,4-thiadiazole -2-thione, potassium salt



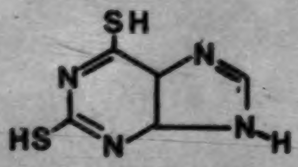
2-mercaptobenzimidazole



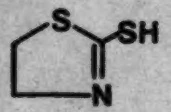
2-mercaptobenzothiazole



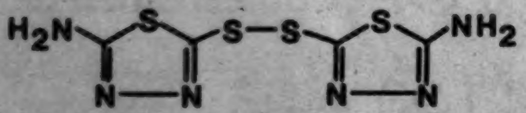
2-mercaptobenzoxazole



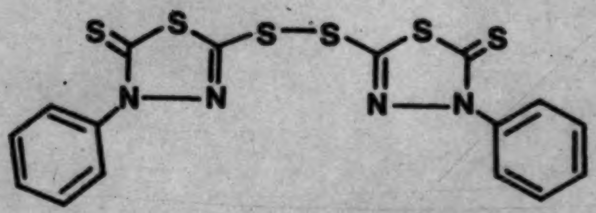
2,6-dimercaptopurine



2-thiazoline-2-thiol

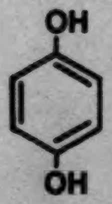


2,2'-di(5-amino-1,3,4-thiadiazole) disulfide

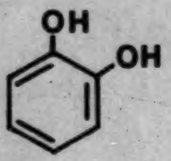

S
S
S
S
S
S
N
N
N
N

5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide

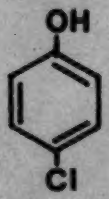
B. Phenols and Benzoquinones



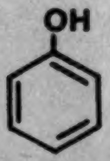
hydroquinone
(1,4-dihydroxybenzene)



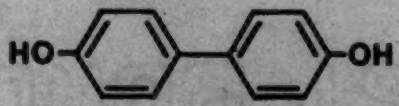
catechol
(1,2-dihydroxybenzene)



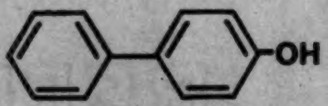
4-chlorophenol



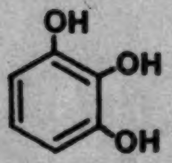
phenol



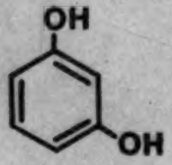
p,p'-biphenol



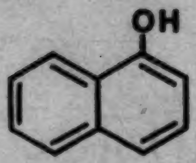
4-phenylphenol



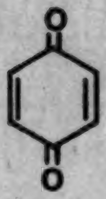
pyrogallol
(1,2,3-trihydroxybenzene)



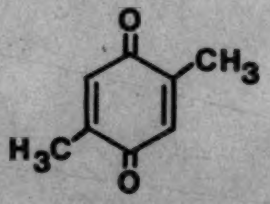
resorcinol
(1,3-dihydroxybenzene)



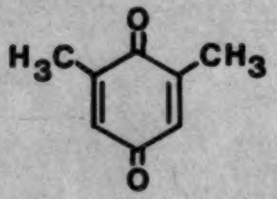
1-napthol



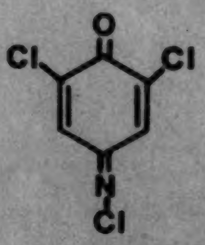
1,4-benzoquinone



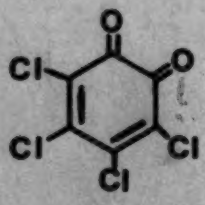
2,5-dimethyl-1,4-benzoquinone



2,6-dimethyl-1,4-benzoquinone

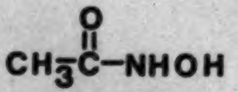


2,6-dichloroquinone-4-chloroimide

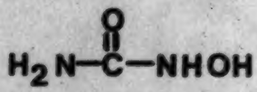


tetrachloro-1,2-benzoquinone

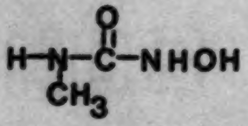
C. Urea Analogs, Thiourea Derivatives, and Hydroxamates


$CH_3\overset{\overset{O}{\|}}{C}-NHOH$

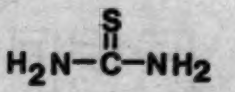
acetohydroxamic acid


$H_2N-\overset{\overset{O}{\|}}{C}-NHOH$

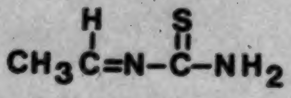
hydroxyurea


$H-\underset{\underset{CH_3}{|}}{N}-\overset{\overset{O}{\|}}{C}-NHOH$

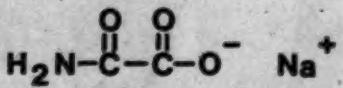
N'-methyl-N-hydroxyurea


$H_2N-\overset{\overset{S}{\|}}{C}-NH_2$

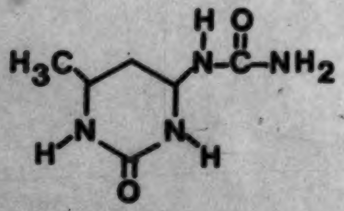
thiourea


$CH_3\overset{\overset{H}{|}}{C}=N-\overset{\overset{S}{\|}}{C}-NH_2$

acetaldehyde thiourea


$H_2N-\overset{\overset{O}{\|}}{C}-\overset{\overset{O}{\|}}{C}-O^- \quad Na^+$

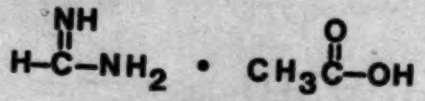
sodium oxamate



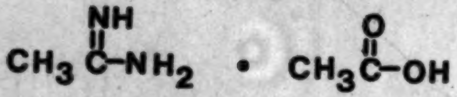
crotonylidene diurea (CDU)

(2-oxo-4-methyl-6-ureidohexahydropyrimidine)

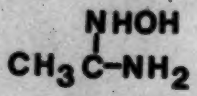
D. Aldoximes and Imides


$$\mathrm{H{-}\overset{\overset{NH}{\|}}{C}{-}NH_2 \cdot CH_3\overset{\overset{O}{\|}}{C}{-}OH}$$

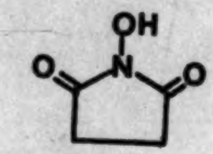
formamidine acetate


$$\mathrm{CH_3\overset{\overset{NH}{\|}}{C}{-}NH_2 \cdot CH_3\overset{\overset{O}{\|}}{C}{-}OH}$$

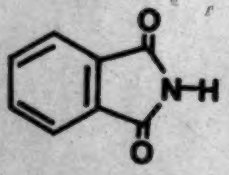
acetamidine acetate


$$\mathrm{CH_3\overset{\overset{NHOH}{|}}{C}{-}NH_2}$$

acetamidoxime

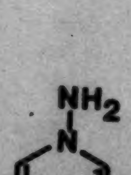


N-hydroxysuccinimide

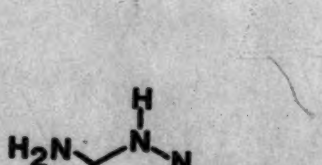


phthalimide

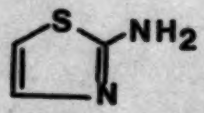
E. Pyridine Derivatives and Heterocyclic Nitrogen Compounds



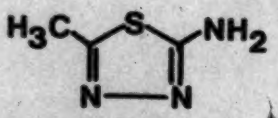
4-amino-1,2,4-triazole



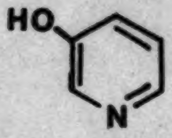
5-aminotetrazole monohydrate



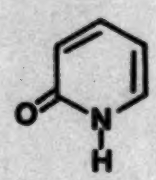
2-aminothiazole



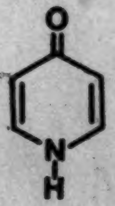
2-amino-5-methyl-1,3,4-thiadiazole



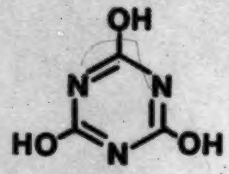
3-pyridinol



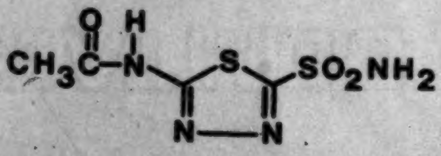
2(1H)-pyridone



4(1H)-pyridone



cyanuric acid



2-sulfonamide-5-acetamido-1,3,4-thiadiazole

Appendix II

Chemical and Physical Analyses of the Soil Used

pH	Total CEC meq/100 g	Total Carbon, %	Mechanical Analysis		
			Sand %	Silt %	Clay %
6.40	37	6.40	29	39	32

Appendix III

Infrared spectrum of 5-amino-1,3,4-thiadiazole-2-thiol in a KBr pellet

Absorption bands, cm^{-1}	Assignment (Colthup, Daly and Wiberley, 1975, Dyer, 1965)
3360 S	NH_2 stretch
3265 S	NH_2 stretch
3070 S	NH_2 stretch
2910 S	N-H stretch*
2770 M	N-H stretch*
1585 S	thiazole ring stretch
1525 S	=N-H bending
1488 S	thiazole ring stretch
1355 S	thiazole ring stretch
1320 S	N-C=S
1280 M	$=C-NH_2$
1045 S	C=S stretching
1015 M	
740 S	=N-H
665 M	C-S stretch
600 W	C-S stretch
552 W	C-S stretch

S = strong M = medium W = weak

* In heteroaromatic compounds the N-H stretch frequency is lowered to 2800-2600 cm^{-1} if there is appreciable hydrogen bonding (Colthup, Daly and Wiberley, 1975).

Appendix IV

Infrared spectrum of 2,2'-di(5-amino-1,3,4-thiadiazole) disulfide in a KBr pellet

Absorption bands, cm^{-1}	Assignment (Colthup, Daly and Wiberley, 1975, Dyer, 1965)
3270 S	NH_2 stretch
3100 S	NH_2 stretch
1638 S	-S-C=N
1500 S	thiazole ring stretching
1385 M	thiazole ring stretching
1315 W	
1128 S	$C-NH_2$
1030 M	$C-NH_2$
675 W	C-S stretch
610 M	C-S stretch
565 M	C-S stretch

S = strong M = medium W = weak

Appendix V

Infrared spectrum of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2-thione in a KBr pellet

Absorption bands, cm^{-1}	Assignment (Colthup, Daly and Wiberley, 1975, Dyer, 1965)
3420 M	C-H stretch (aromatic)
2330 W	S-H stretch
1640 W	S-C=N
1585 W	ring stretch (aromatic)
1490 S	ring stretch (aromatic)
1480 M	thiazole ring stretching
1450 W	ring stretch (aromatic)
1420 W	
1345 S	thiazole ring stretching
1290 W	
1230 S	ring stretch (aromatic)
1065 M	C-H bend (aromatic)
1030 S	C=S stretching
835 M	C-H bend (aromatic)
755 M	C-H wag (aromatic)
690 M	C-H wag (aromatic)
680 M	C-S stretch
600 W	C-S stretch

S = strong M = medium W = weak

Appendix VI

Infrared spectrum of 5,5'-di(3-phenyl-1,3,4-thiadiazole-2-thione) disulfide in a KBr pellet

Absorption bands, cm^{-1}	Assignment (Colthup, Daly and Wiberley, 1975, Dyer, 1965)
3430 S	C-H stretch (aromatic)
1585 M	ring stretch (aromatic)
1485 S	thiazole ring stretch
1320 M	thiazole ring stretch
1215 S	ring stretch (aromatic)
1030 M	C=S stretching
900 W	C-H bend (aromatic)
812 M	C-H bend (aromatic)
752 M	C-H wag (aromatic)
695 M	C-H wag (aromatic)
680 S	C-S stretch
640 W	C-S stretch
590 W	C-S stretch

S = strong M = medium W = weak